Role of Aryl Hydrocarbon Receptor (AHR) and the effect of Selective AHR Modulators (SAHRMs) on T cell differentiation and effector function

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By

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Role of Aryl Hydrocarbon Receptor (AHR) and the effect of Selective AHR Modulators (SAHRMs) on T cell differentiation and effector function

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A number of autoimmune and chronic inflammatory diseases are related to environmental stress. Aryl hydrocarbon receptor (AHR) is regarded as an environmental sensor integrating immune responses with environmental stress and thereby influencing the extent of host immune response. The physiological role of AHR, particularly, in the immune response remains a key question after the discovery that AHR can modulate Th17/Treg axis in a ligand specific manner. However, discovery of a non-DRE mediated AHR activation in addition to its canonical DRE-mediated pathway further adds to this complexity thereby necessitating a more critical approach in delineating the role of AHR in the context of Th17/Treg balance. Selective AHR modulators (SAHRMs) are a recently identified class of compounds that are capable of binding to AHR and repress cytokine- driven gene expression without activating DRE-driven responses We have used three classes of AHR ligands namely agonist, antagonist and partial antagonist (SAHRM) to delineate the role of AHR in the context of Th17/Treg regulation both *in vitro* and *in vivo*. Here, we show that inhibition of the DRE-driven response is sufficient to suppress the Th17 differentiation. Also, we show that the suppression of Treg differentiation is dependent on the inhibition of the non-DRE mediated pathway. We

have also delineated the role of DRE vs non-DRE driven responses of Th17 regulation in both an acute vs a chronic Th17 mediated diseases. In an acute inflammation using *C. rodentium* model of IBD (Inflammatory Bowel Disease) AHR agonist enhanced Th17 production and thereby promoted resolution of infection. On the other hand, using *S. rectivirgula* in a chronic mouse model of Hypersensitivity Pneumonitis we show that inhibition of DRE and /or non-DRE mediated response had minimal effect on IL-17A production but precludes Th17 plasticity by enabling secretion of alternate lineage anti-inflammatory cytokines. This work has profound implication in pharmacological intervention in autoimmune and chronic inflammatory diseases by developing AHR compounds which can modulate DRE and non-DRE driven responses.

BIOGRAPHICAL SKETCH

Sonia got interested in biological sciences from an early stage during her school days. She opted for a Masters in Biotechnology in India and came to United States to pursue a PhD in Molecular Oncology. During her coursework she got interested in immunology and got a Masters in Molecular Oncology and decided to pursue a doctoral degree under Dr Avery August. During her stay in August-lab she got exposed to a broad range of research topics and opportunities to learn a wide array of cutting edge technologies in this field. Her main objective of graduate study is to acquire an in-depth knowledge in the field of T cell biology and eventually carve a niche as an independent researcher in this field. The primary focus of her research is to understand the intricate process that governs the link between environmental stress and autoimmune/chronic inflammatory diseases by studying the role of an environmental sensor molecule in the context of immune response. The thorough training in her graduate studies helped her to cultivate technical skills, enrich her scientific knowledge and lay a strong foundation for her future career path in immunology. She considers herself fortunate to work in a lab best suited to accomplish her endeavor to become an independent researcher in the field of immunology. She intends to receive a post-doctoral training following her completion of doctoral program in the field of autoimmune and chronic inflammatory diseases to carve a niche as an independent researcher in this field.

DEDICATION

To my Parents

Lab Kumar Mohinta and Mithu Mohinta

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I would like to thank my mentor Dr Avery August for his support and guidance throughout the project. As a mentor he is extremely devoted and in the lab he is always open to questions or research ideas. I would just walk in and talk to him and he would always be receptive which helped me immensely to grow as an independent researcher. I would like to thank all the members of August lab for their support and help. I am grateful to my collaborator Dr Gary Perdew for always being supportive and providing me with the AHR compounds. I would like to thank members of my graduate thesis committee, Dr Scott Coonrod, Dr Eric Denkers, and Dr Margaret Bynoe for their support and guidance. I would also like to thank the M&I staff members for help with technical and administrative issues. I would like to thank all my friends for being there for me when I needed. I am grateful to Dr Arun Kannan for always encouraging and supporting me, Dr Krishna Jhaveri, Dr Debashree Mukherjea and Dr Vickram Ramkumar for helping me out during my stay at Springfield, IL.

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Chapter 1

Introduction

1.1 Introduction

The aryl hydrocarbon receptor (AHR) is a highly conserved molecule and its functional orthologues are found throughout a number of animal species from mammals, amphibians and reptiles to aves suggesting its importance in the animal kingdom. Studies on AHR for the past 50 years have revealed its role as a sensor for environmental toxin and a mediator of chemical toxicity. Historically, immunotoxicity of AHR was deduced from studies of halogenated aromatic compounds (HAH) and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD; ‘‘dioxin’’), the most toxic ligand that binds to AHR with high affinity. AHR was originally discovered as a transcription factor that mediated cellular toxicity by upregulating xenobiotic enzymes through P450 cytochrome (Cyp1A) genes. Studies with mouse Cyp1A1 gene expression identified enhancer sequences5’-TNGCGTG-3’ on AHR target genes referred to as xenobiotic response elements (XRE) or dioxin response elements (DRE). AHR has been widely studied in the context of TCDD mediated toxicity, which induces liver damage, thymic involution, epithelial hyperplasia and immune dysregulation. AHR is expressed in lung, heart, thymus, liver and placenta essential for diverse roles in these organs. Recently, AHR has been shown to play a critical role in the homeostasis of the immune system.

1.2 Structure of AHR

AHR belongs to Per-ARNT-Sim (PAS) superfamily of proteins. General domain organization of AHR consists of an N-terminal basic helix-loop-helix (bHLH) domain, a C-terminal variable domain and a central PAS domain which consists of 260-310 amino acids and contains two conserved hydrophobic repeats PAS-A and PAS-B which mediate a number of diverse functions including binding to AHR specific ligand, and heterodimerization with aryl hydrocarbon translocator (ARNT) to form the active transcription factor that translocates to the nucleus and interacts with other chaperone proteins. The basic helix-loop-helix (bHLH) domain is also involved in DNA binding and heterodimerization. The variable C-terminal domain contains the transactivation domain (TAD), which mediates transcription after target DNA binding (**Fig.1**).

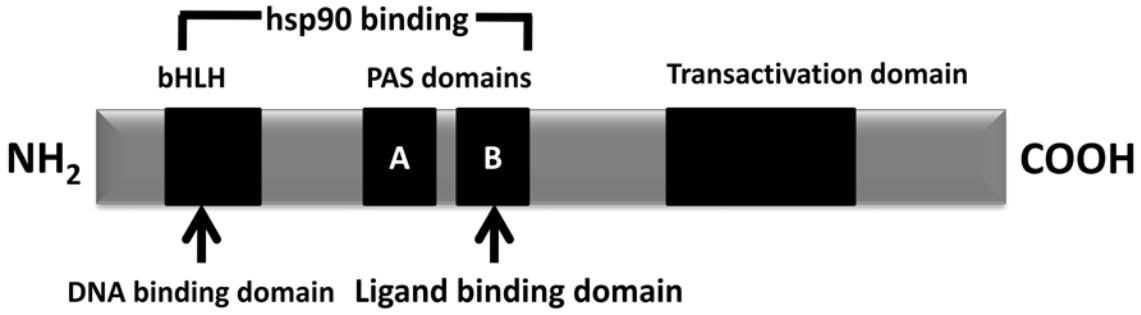

Fig.1. Structure of AHR. Model diagram depicting the domain organization of AHR. AHR consists of a highly conserved N-terminal domain containing the bHLH, a central hydrophobic region containing the PAS-A and PAS-B domains and a poorly conserved C-terminal domain containing the transactivation domain which is required for

transcription after target DNA binding. AHR binds to PAH (polycyclic aromatic hydrocarbons), HAH (halogenated aromatic hydrocarbons) and also non-aromatic compounds by binding to the PAS-B domain.

1.3 Canonical AHR pathway

AHR is a cytosolic transcription factor belonging to the basic helix-loop-helix (bHLH)- Per-ARNT-Sim (PAS) family and is evolutionarily conserved across a number of species in animal the kingdom [1]. In absence of a ligand, AHR remains complexed with heat shock protein 90 (hsp90) [2, 3],immunophilin-related protein (XAP2) [4, 5] and phosphoprotein p23 [6]. Upon ligand binding AHR undergoes conformational changes and exposes the nuclear localization signal (NLS) and translocates to the nucleus from the cytosol [7]. In the nucleus it dissociates from the cytosolic proteins and dimerizes with ARNT[8] to adopt an active form of transcription factor that can bind to the specific DNA sites (xenobiotic response elements XRE) with high affinity and modulate transcription of AHR responsive genes (**Fig2**). AHR has been mainly studied as a mediator of dioxin toxicity [9, 10] but recent studies have shown that it plays an important role in the development of liver [11], vascular system [12] and modulates signaling in immune cells in response to environmental toxins.

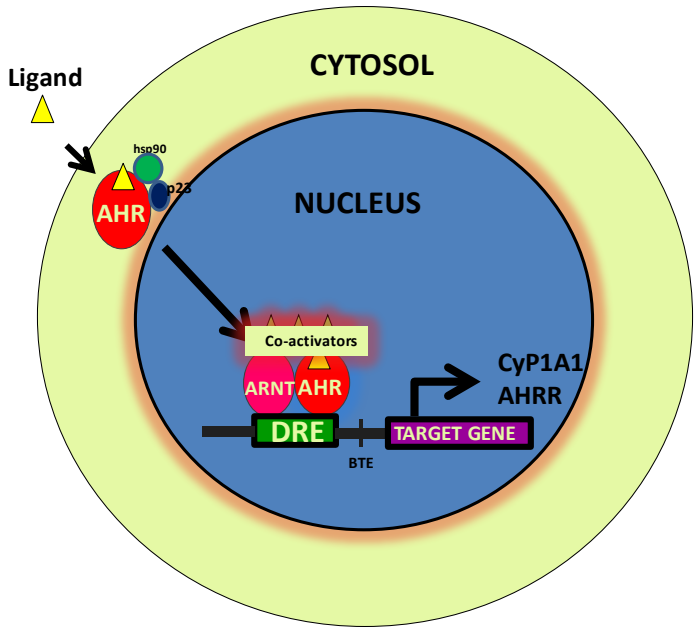

Fig. 2. The canonical AHR pathway. Model diagram depicting the AHR canonical pathway. AHR remains complexed with heat shock protein (hsp90) and p23 in absence of ligand binding in the cytosol. Upon ligand binding it heterodimerizes with AHR-nuclear translocator (AHRNT) and is transported to the nucleus and initiates transcription of its target genes. One of the most potent genes induced by AHR activation is Cyp1A1, a p450 cytochrome required to detoxify xenobiotics.

1.4 Defects in AHR-null mice

AHR knockout mice provide a good understanding of the physiological role of AHR. AHR deficient mice have been shown to have a myriad of defects in hepatic, vascular and hematopoietic development along with a lower life expectancy and reduced reproductive capacity. In liver, AHR is required to close the ductus venosus after birth and its deficiency causes impaired blood flow in the liver along with portal tract fibrosis that result in a significantly smaller liver. AHR deficiency also leads to a number of reproductive defects including death of females during pregnancy and defects in lactation that may be due the inability to sufficiently synthesize hormones,

sudden death of pups and smaller litter size. In the immune system AHR deficiency leads to decreased lymphocytes in spleen and have reduced ability to maintain compartments of hematopoietic stem cells.

1.5 AHR in T cell biology

$CD4^+$ T cells play a central role in adaptive immune response (**Fig3**). They are capable of exerting a myriad of functions which ranges from helping B cells to generate antibody and regulating $CD8^+$ T cells responses, to inducing immune response to a variety of pathogenic microorganisms, regulating and fine tuning autoimmune responses, and establishing immunologic memory. Following their initial characterization in 1986 by Mosmann and Coffman into two distinct subsets of Th1 and Th2 lineages, a number of distinct $CD4^+$ T cell subsets have emerged. Th17 and Treg cells are two such lineages that have well characterized in recent times [13, 14]. These subsets are distinguished by their cytokine profile and effector function. Th1 cells secrete IFN-γ and lymphotoxin in response to intracellular pathogens. Th2 cells produce IL-4, IL-5 and IL-13 to mediate immune response to extracellular pathogen. In response to extracellular bacteria and fungi Th17 cells secrete pro-inflammatory cytokines like IL-17A, IL17F, IL-21 and also IL-22 [14, 15]. Tregs on the other hand secrete anti-inflammatory cytokines and mediators and are critical for control of inflammatory and autoimmune responses.

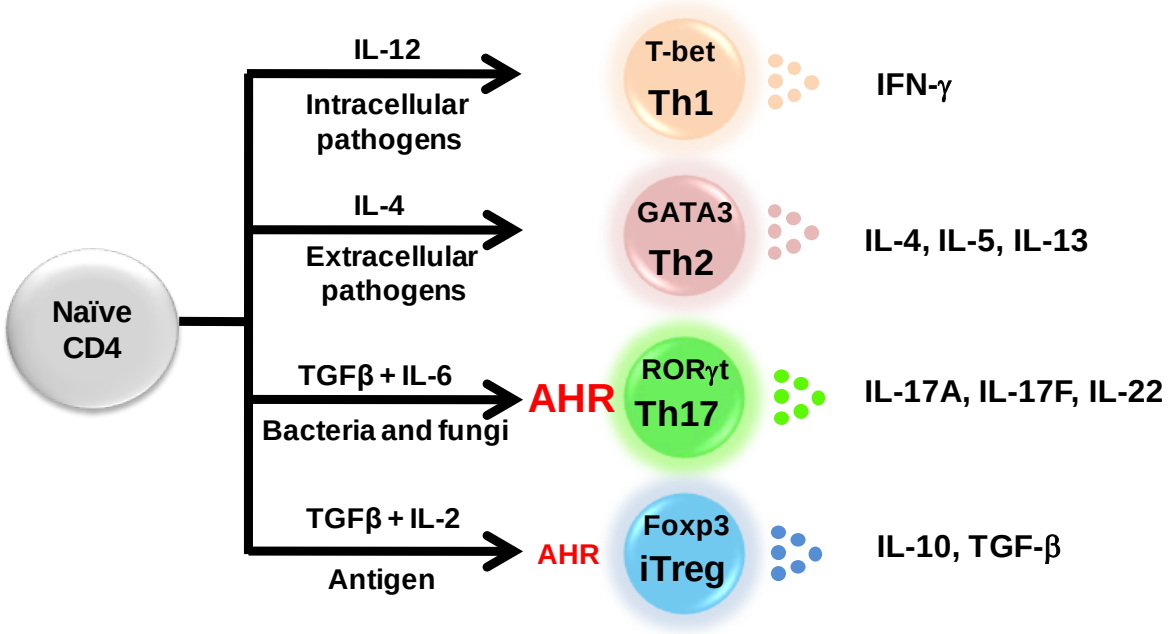

Fig. 3. CD4$^+$ T cell subsets and their cytokines. Upon antigenic stimulation a naïve CD4$^+$ T cell can give rise to at least 4 different subtypes of T helper cells. In presence of IL-12 and intracellular pathogens like bacteria it can differentiate to Th1 cells by upregulating T-bet and secrete IFN-γ and in presence of IL-4 and extracellular parasites it can differentiate to Th2 cells by upregulating GATA3 which can secrete IL-4, IL-5 and IL-13. In presence of TGF-β and IL-6 and extracellular bacteria and fungi a naïve CD4$^+$ T cell differentiates to Th17 cells by upregulating RORγt and secrete cytokines like IL-17A, IL-17F and IL-22. Tregs are induced peripherally in the presence of above mentioned antigens and TGF-β to upregulate Foxp3 and can secrete immunosuppressive cytokines like TGF-β and IL-10. AHR is expressed in both Th17 and Treg cells and can promote their differentiation in a ligand specific manner.

The IL-17 family of cytokines includes IL-17A, IL-17F, IL-17B, IL-17C, IL-17D and IL-17E (IL-25). IL-17A and IL-17F share the highest structural homology (50%) and have a number of similar functional properties. The IL-17 receptor family includes 5 members from IL-17RA to IL-17RE and homodimers or heterodimers of these

receptors bind to IL-17 family cytokines [16]. IL-17A homodimers or Il-17A-Il17F heterodimers binds to the heterodimer of IL-17RA and IL-17-RC. With the exception of IL-17E, all members of the IL17 family of cytokines induce expression of proinflammatory cytokines like IL-1β and tumor necrosis factor (TNF) by other cell types and also promote neutrophil migration suggesting a common role for these cytokines in certain inflammatory diseases [14, 16]. A naïve $CD4^+$T cell differentiates into Th17 cells in presence of TGF-β1 and IL-6 or IL-21 by upregulating RORγt, a master regulator of Th17 cell differentiation. IL-6 or IL-21 alone cannot drive the differentiation of Th17 cells in the absence of TGF-β1 which is responsible for dampening the Th1 and Th2 responses [17]. However recently, it has been shown that this requirement for TGF-β1 can be overcome by IL-1β in the presence of either IL-6 or IL-21 [18]. It is becoming increasingly clear that a number of inflammatory and autoimmune diseases that were initially thought to be mediated by Th1 are actually driven by an over-exuberant Th17 response. Different disease models including experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis (CIA) and autoimmune myocarditis are directly linked with IL-17A cytokine as neutralizing this cytokine with IL-17A specific antibodies can block the initiation of the disease [17].

Regulatory T cells (Tregs) are another subset of $CD4^+$T cells, which plays an important role in the suppression of autoimmune diseases, minimize the deleterious effects of pathogen, maintain self-tolerance and control the exacerbation of inflammatory processes [19]. Foxp3 expression is a hallmark of $CD4^+$ Tregs. TGF-β1 along with IL-2 induces the differentiation of Treg from naïve $CD4^+$ T cells. Tregs

can suppress inflammatory responses in a number of ways including secretion of inhibitory cytokines like TGF-β and IL-10, direct cytolysis through secretion of granzymes A/ B, metabolic disruption and by inhibition of dendritic cell activation and function [19] Thus, Tregs play a critical role in immune cell homeostasis and TGF-β1 can induce differentiation of both Th17 and Treg cells. In fact, T cell activation in the presence of TGF-β1 induces expression of both RORγt and Foxp3. Foxp3 suppresses RORγt by directly binding to it during Treg cell differentiation while Foxp3 is suppressed under Th17 polarizing conditions [20]. Suppression of RORγt by Foxp3 can be overcome by cytokines that signal through Stat3 like IL-6, IL-21 and IL-23 [20]. Thus the Th17/Treg balance is carefully controlled by a dynamic interplay between cytokines and the downstream transcription factors they induce [21].

In recent times, activation of AHR by its ligand has been shown to influence differentiation of different subsets of T helper cells. An orally active anti-allergic AHR agonist M50367 has been shown to regulate the Th1/Th2 cell axis. Mice treated with this agent in an allergic experimental model had decreased response via suppression of IgE and peritoneal eosinophilia resulting in reduced airway hyperresponsiveness. M50367 skews naïve $CD4^+$ T cells to a Th1 phenotype, which is mediated by the suppression of Th2 master transcription factor GATA3, leading to a decreased allergic activity. These results suggest that AHR is an attractive therapeutic target for the treatment of allergic diseases.

The most potent ligand of AHR, TCDD has profound effects on both humoral and cellular immunity. TCDD is unaffected by the metabolizing enzymes induced by AHR activation and its persistence in the cellular milieu is the primary reason for its potent toxicity [22]. TCDD exposure causes thymic atrophy by targeting the intrathymic progenitor cells [23],acute thymocyte cell loss and proliferation arrest [24] and preferential thymic egress of DN thymocytes in the periphery [25]. Recently, two groups have reported a dichotomous behavior of AHR agonists. Quintana *et al* showed that activation of AHR by TCDD led to the induction of functional Treg cells that suppressed experimental autoimmune encephalomyelitis or EAE in a TGF-β1-dependent mechanism. In line with this, Kimura *et al* using AHR deficient animals showed that AHR signaling can promote the differentiation of Treg cells. But another group recently showed that, activation of AHR by 6-formylindolo[3,2-b]carbazole (FICZ), another agonist ligand of AHR, results in the induction of TH17 differentiation leading to the worsening of EAE disease pathology (**Fig. 4**).

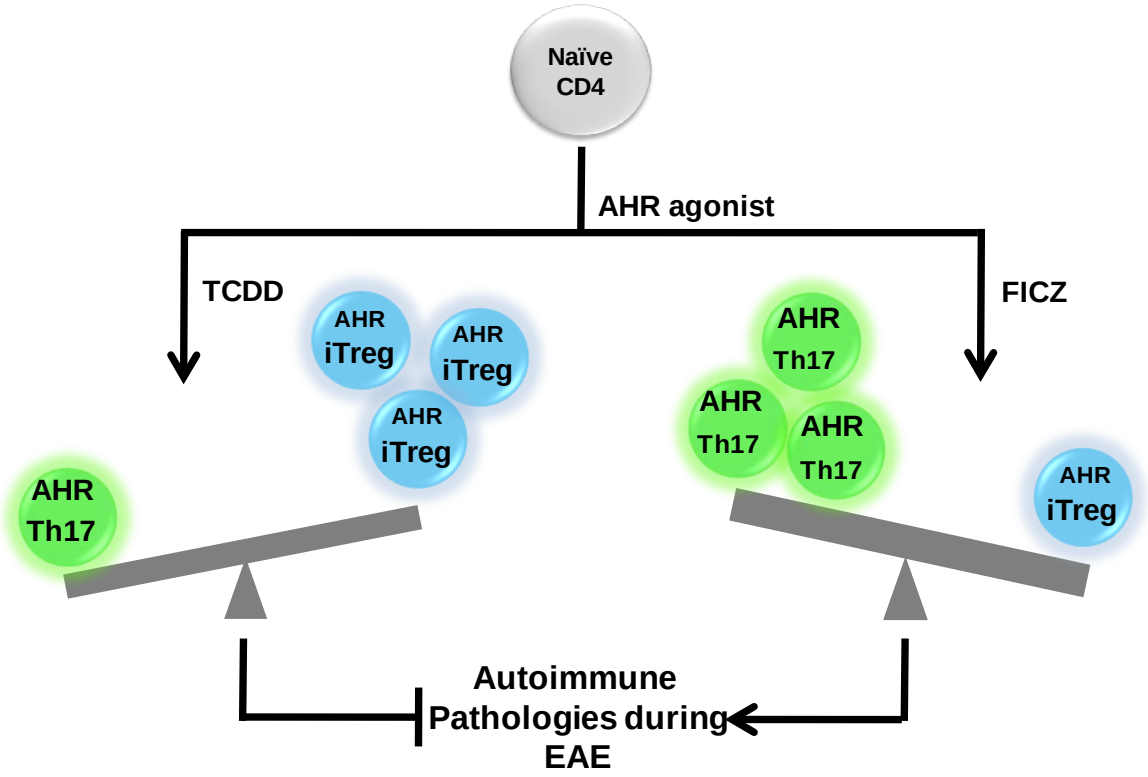

Fig. 4. AHR regulates Th17/Treg axis in a ligand specific manner. AHR binds to TCDD, an agonist, and promotes Treg differentiation thereby protecting mice from EAE whereas treatment with FICZ, also an agonist promotes Th17 differentiation resulting in exacerbation of EAE.

Th17 cells are required to clear microbial pathogens and maintain barrier function but inappropriate Th17 responses mediate autoimmune diseases. AHR expression is significantly higher in Th17 cells compared to Treg cells, and AHR activation leads to both expansion and increased cytokine production by differentiated Th17 cells although it seems dispensable for Th17 differentiation under certain experimental conditions. In line with this, Veldhoen *et al* showed that FICZ activated AHR under Th17 polarizing condition (IL-6 and TGF-β1) *in vitro* to induce the

differentiation of naïve $CD4^+$ T cells to a Th17 phenotype marked by up-regulated IL-17A, IL-17F and IL-22 mRNA expression, sufficient to exacerbate EAE *in vivo* [26]. AHR-deficient mice on the other hand under similar conditions had attenuated IL-17A and IL-17F secretion with no detectable IL-22 in presence or absence of FICZ [26]. Although these results have highlighted the essential role of AHR in Treg and TH17 cells, the mechanism by which AHR mediates this effect is largely unknown. This apparent paradox between FICZ and TCDD exposure in EAE suggests that AHR is activated in a ligand specific manner which differentially regulates Th17 vs Treg differentiation [27]. Thus, AHR fine-tunes the Treg/Th17 balance based on the cytokine milieu in the cellular microenvironment in a ligand specific manner. Also, silencing of the IL-2 locus is critical for development of Th17 cells, and it has been shown that under Th17 polarizing conditions this is mediated by induction of Aiolos, a member of Ikaros family of transcription factors, by Stat3 and AHR. In addition to this, activation of AHR in dendritic cells by an endogenous AHR ligand 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) induces tolerogenic dendritic cells in a retinoic acid dependent manner to promote differentiation of $Foxp3^+$ Treg cells. Environmental factors are linked to autoimmune disorders and involvement of AHR in Treg/Th17 differentiation and function opens up new avenues for the treatment of these disorders.

1.6 Identification of a DRE-independent pathway

Recently, it was shown that AHR can regulate NFκB regulated gene expression especially acute phase genes such as serum amyloid A (Saa). Acute phase genes are induced due to environmental stress, inflammation or neoplastic growth and a sustained induction is unwarranted and deleterious. Although acute phase proteins (APP) like serum amyloid A, plasminogen and C-reactive protein (CRP) that are made in liver are required as a first line of defense, in chronic inflammatory settings such as autoimmune diseases these proteins can have detrimental effects on immune signaling, amylodoisis and catabolism. AHR activation by certain ligands has been shown to suppress Saa, which are induced in response to cytokines in liver, suggesting AHR is a potential therapeutic target for treatment of chronic inflammatory/autoimmune diseases. However, ligand bound AHR requires nuclear translocation with AHR-nuclear translocator and heterodimerization but it does not require DNA binding to suppress Saa expression. Thus activation of AHR bypassing the DRE-mediated DNA binding pathway can lead to the suppression of acute phase genes, which opens up new avenues in the treatment of chronic inflammatory conditions, like cancer and autoimmune diseases.

1.7 Why study Selective AHR modulators (SAHRMs)

Selective AHR modulators (SAHRMs) are a recently identified class of compounds that are capable of binding to AHR without activating DRE-driven responses. An agonist activates both DRE and non-DRE pathways while an antagonist inhibits both

of them but a partial antagonist It selectively inhibits only the DRE- mediated pathway while the non-DRE driven responses remain unaffected (**Fig.5**). These compounds are interesting therapeutically as they can potentially be used to inhibit DRE-mediated transcription while inducing anti-inflammatory or other non-DRE AHR dependent responses. A number of reports have highlighted the adverse effect of a sustained AHR activation with AHR agonists, which results in increased inflammation. However, recently it was shown that AHR activation can also lead to anti-inflammatory responses by interacting with NFκB signaling or suppressing proinflammatory cytokines like IL-6 and this pathway is independent of its ability to bind DNA (DRE-mediated pathway), a non-DRE-mediated/non-canonical AHR pathway. Since, sustained activation of AHR by DRE-mediated pathway leads to a toxic response, activation of AHR without affecting DRE-mediated responses can be beneficial as it can suppress the induction of acute phase genes without cellular toxicity. The potential role of SAHRMs is gaining momentum due to the recent findings that AHR can be modulated to affect disease pathophysiology. Firstly, suppressing AHR by AHR antagonist can inhibit Th17 cell development, which is the prime mediator of autoimmune diseases like rheumatoid arthritis and multiple sclerosis. Secondly, as aforementioned, activation of AHR via the non-canonical pathway bypassing the DRE- mediated pathway can lead to the suppression of acute phase genes like Saa that promote inflammation and amyloidosis, and lastly modulating AHR selectively can also affect estrogenic activity in breast cancer patients. Hence, SAHRMs are an attractive strategy to modulate AHR for treatment of these chronic diseases. However, an agonist will globally activate AHR through

the activation of DRE-mediated pathway leading to a toxic response, while a complete antagonist will suppress both the deleterious effects of DRE activation and the beneficial anti-inflammatory effects of the non-DRE mediated pathway.

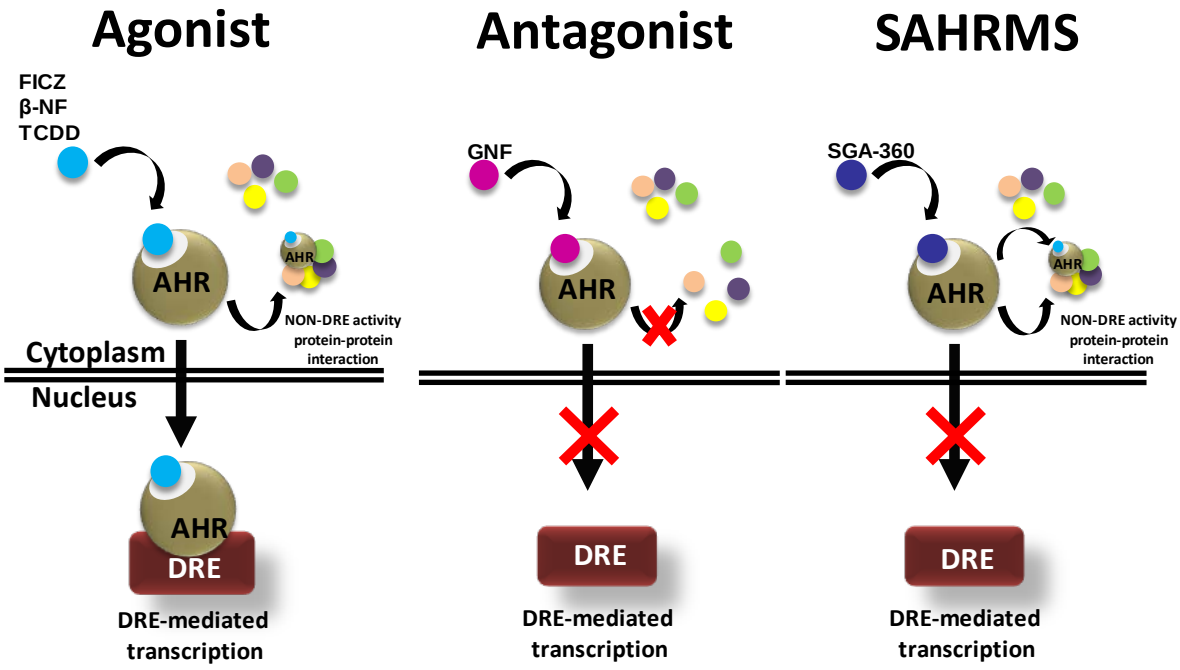

Fig. 5. Activation or inhibition of DRE and non-DRE mediated AHR pathway. AHR can be activated by both DRE and non-DRE mediated pathways by an AHR agonist whereas a complete antagonist would inhibit both. The SAHRMs on the other hand can selectively activate AHR only by the non-DRE mediated pathway but inhibits the DRE-driven responses.

In this study we have explored potential of AHR modulation by selective or global activation by SAHRMs and agonists respectively as well as inhibition of AHR by a complete antagonist in regulation of T helper cell responses. This thesis is driven by the ***central hypothesis*** that regulating AHR with AHR ligands can modulate Th17 versus Treg differentiation to control pro- versus anti-inflammatory responses *in*

vivo. We will achieve this by working on the following specific aims**: 1. Determine the role of the AHR in T cell differentiation by using AHR ligands that modulate the AHR pathway.** We ***hypothesize*** that the selective AHR modulators (SAHRMs) will modulate AHR activity and affect T cell differentiation. Our preliminary data suggests modulating AHR with SAHRMs in $CD4^{+}$T cells affects Th17 and Treg differentiation *in vitro*. **2. Determine the effect of AHR modulation by SAHRMs on T-helper cell plasticity *in vivo*.** We ***hypothesize*** that the terminally differentiated T-helper cells will be skewed in the presence of SAHRMs.

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CHAPTER 2

Role of aryl hydrocarbon receptor (AHR) and the effect of selective AHR modulators (SAHRMs) on T cell differentiation

2.1 Introduction

A naïve $CD4^+$ T cell upon antigenic stimulation can differentiate into at least four different subset of T helper lineages namely Th1, Th2, Th17 and Treg cells [1-3]. Th1 cells are characterized by secretion of IFN-γ upon stimulation by intracellular antigens and the Th1 lineage program is regulated by the master transcription factor T-bet. Extracellular parasites induce naïve $CD4^+$ T cells to upregulate GATA3 and differentiate Th2 cells that can secrete IL-4, IL-5 and IL-13. Th17 cells are induced by extracellular bacteria and fungi to secrete IL-17A or eponymous cytokine IL-17F along with upregulation of RORγt [4]. Treg cells on the other hand suppress aberrant immune responses by expressing Foxp3 and secreting anti-inflammatory cytokines like IL-10 and TGF-β[5].

The aryl hydrocarbon receptor (AHR) is a type I nuclear receptor that is expressed in both Th17 and Treg albeit at higher proportion in Th17 than in Tregs or any other Th cell subset. AHR agonist TCDD or dioxin promotes differentiation of $CD25^+Foxp3^+$Treg cells and suppresses EAE, whereas the tryptophan photoproduct 6-formylindolo[3,2-b] carbazole (FICZ), an endogenous ligand of AHR, induces IL-17A resulting in exacerbation of EAE [6-8]. However, in AHR knockout mice there was minimal defect in generation of Treg or Th17 cells but Th17 cells were defective in the secretion of IL-22, a cytokine co-expressed with IL-17A and is required for mucosal immunity and secretion of anti-microbial peptides [4, 6, 7, 9]. A better understanding of this apparent dichotomous behavior of AHR ligands could be useful in identifying links between environmental ligands of AHR, including toxins, and the development of autoimmune

diseases. AHR may be activated via two pathways, a DRE- mediated DNA binding pathway or via non-DRE mediated protein-protein interaction [10-12]. Based on how AHR is activated, it can result in very different outcomes [13-19]. An agonist would activate both DRE and non-DRE pathways, whereas an antagonist inhibits both [10, 12]. On the other hand, a partial antagonist like SGA-360 would selectively inhibit the DRE- driven downstream effects and activate the non-DRE mediated pathway. Recently, it has been shown that selectively activating AHR by inhibiting the DRE-driven activity can be beneficial in repression of cytokine mediated acute phase genes like serum amyloid A (saa) which can be detrimental in chronic inflammatory diseases like rheumatoid arthritis [11, 20, 21].

Selective AHR modulators (SAHRMs) are a recently identified class of compounds that are capable of binding to AHR, activating non-DRE-driven responses without inhibiting the DRE driven responses [11]. These compounds are interesting therapeutically as they can be used to inhibit the DRE-mediated pathway while promoting other functions of the AHR, including anti-inflammatory responses through the non DRE-mediated pathway [11]. It has been shown that activation of naïve $CD4^+$ T cells under Th17 inducing conditions strongly upregulated the expression of AHR [6, 8]. In this study, we have used a partial/selective antagonist called SGA-360, which blocks the DRE function of AHR while selectively activating the non-DRE functions of AHR [11]. We have also examined the functions of other complete agonists of AHR, TCDD, FICZ and β-NF (beta-naphthoflavone) [6-8, 22] . We have also used a complete antagonist, GNF, which antagonizes both the DRE-mediated and acute-phase gene repression activities of AHR [12]. Here we show that, agonist and antagonist ligands of AHR differentially affect the

differentiation of Th17 and Treg cells. More importantly, the effect of agonist on differentiation of Th17 and Treg cells was ligand specific. We also find that, under Th17 polarizing conditions, IL-17A expression under Th17 polarizing condition was promoted by the activation of AHR through the canonical AHR pathway while suppression of the non-canonical non-DRE mediated AHR pathway resulted in the suppression of Foxp3 under Treg skewing conditions.

2.2 Results

2.2.1 Selective inhibition of the DRE pathway is sufficient to inhibit IL-17A

To determine the effect of AHR ligands on the differentiation of effector $CD4^+$ T cells *in vitro* we stimulated splenocytes from OTII mice (transgenic mice in which $CD4^+$ T cells express a TCR which can recognize chicken Ovalbumin) with Ovalbumin (OVA) peptide (OVA 323-339) in the presence of SAHRM SGA-360, a partial antagonist, or β-NF, an AHR agonist. The addition of SGA-360 under non-skewing Th0 condition, suppressed the induction of *Cyp1A1*, a known downstream target of AHR [23]. Inhibition of DRE activity of AHR by SGA-360 resulted in an increase in the expression of *IL-4* but did not alter the expression of *IL-17A* or the Th1, Th2 and Th17 lineage defining transcription factors *T-bet, Gata3* and *RoRγt.* On the other hand, under similar conditions β-NF enhanced *Cyp1A1*, *IL-17A, RoRγt,* and suppressed *IL-4* and *Gata3* gene expression (**Fig.1A**). In line with this, β-NF increased IL-17A and suppressed IL-4 cytokine secretion, in contrast to SGA-360, which suppressed the secretion of IL-17A and IFN-γ while inducing the production of IL-4 (**Fig.1B**). This suggests that modulating AHR activity by SAHRMs during T cell activation can differentially affect their ability to secrete effector cytokines, for instance the ability of $CD4^+$ T cells to make IL-17A upon activation even in the absence of Th17 polarizing conditions.

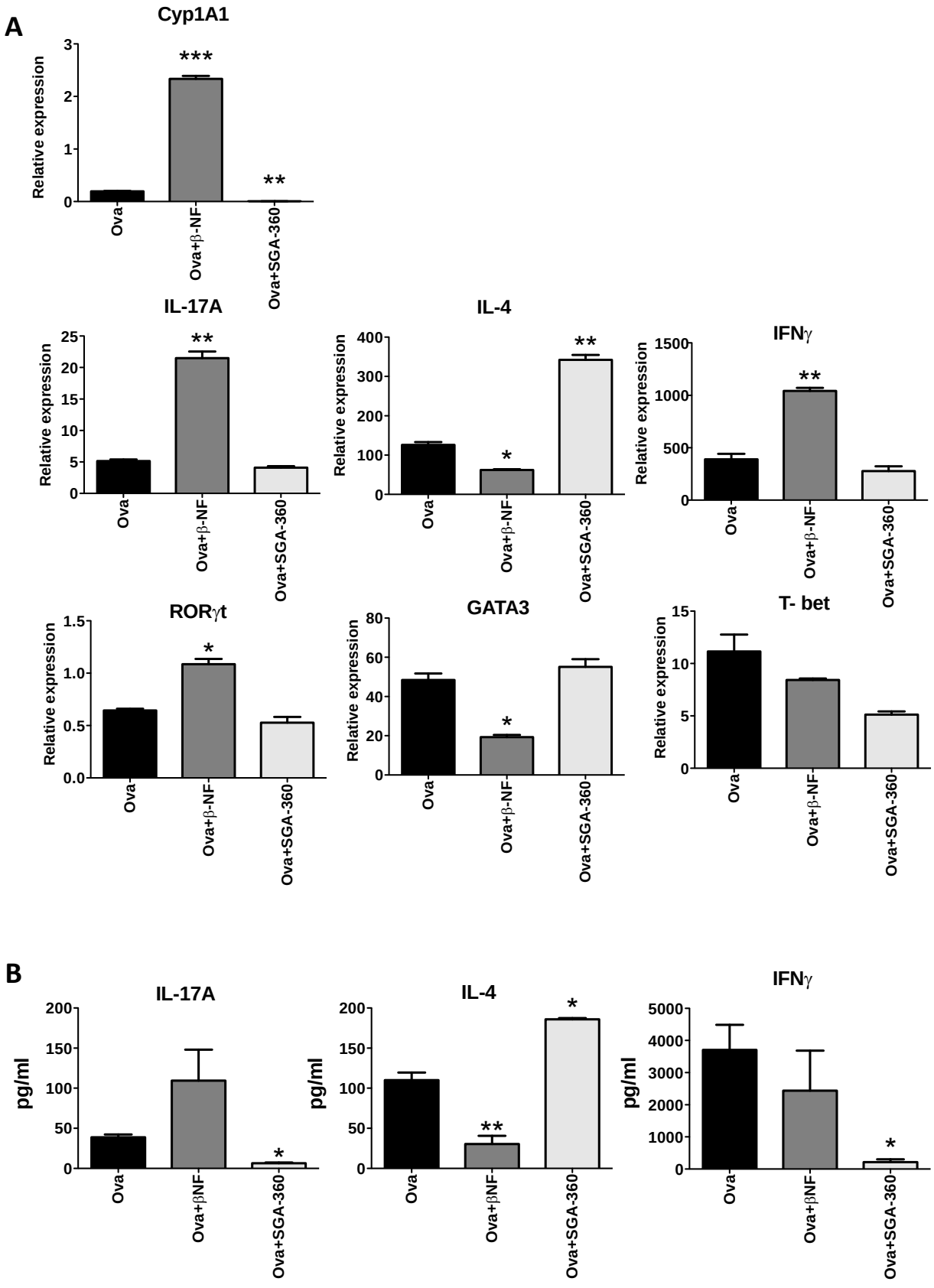

A
Cyp1A1
Relative expression
***
**
Ova
Ova+β-NF
Ova+SGA-360
IL-17A
IL-4
IFNγ
RORγt
GATA3
T- bet
B
IL-17A
IL-4
IFNγ
pg/ml
Ova+βNF

Fig. 1. Selective inhibition of the DRE pathway is sufficient to inhibit IL-17A. A) qPCR analysis of WT OTII splenocytes and lymph node cells that were stimulated with OVA-peptide (323-339) in IMDM media for 5 days in the presence of β-naphthoflavone (2 μM) or SGA-360 (2 μM) and analyzed for the indicated genes. B) Supernatants from cells treated as in A was analyzed for the indicated cytokines by multiplex cytokine assay. Data represents Mean±SEM of n≥3 mice, *p<0.05 by paired students *t* test.

2.2.2 AHR ligands influence Th17 differentiation via the DRE-driven pathway

It has been shown that AHR is induced during Th17 polarizing conditions and AHR is known to act as a regulator of Th17 differentiation in a ligand specific manner [6, 7, 13]. We compared the effect of AHR agonists β-NF, FICZ, TCDD and antagonist GNF along with SAHRM, SGA-360 under Th17 polarizing condition to determine their ability to modulate $CD4^+$ T cell differentiation into Th17 cells. To this end, we isolated naïve $CD4^+$ T cells from IL-17A-GFP reporter mice (transgenic mice containing an IRES-EGFP sequence inserted downstream of the stop codon of IL-17A gene) and stimulated them under Th17 polarizing conditions with IL-6 and transforming growth factor (TGF-β). This reporter model is a useful tool to evaluate the differentiation of Th17 cells (percentage of GFP^+ cells) as well as the amount of IL-17A produced on a per cell basis (mean fluorescence intensity [MFI] of GFP among GFP^+ cells). AHR agonist, FICZ induced both differentiation of Th17 cells and production of IL-17A. β-NF could affect only the frequency of Th17 cells and TCDD only the MFI of IL17-A. These results suggests that the activation of DRE- mediated pathway to modulate the frequency of $CD4^+$ Th17 cells or their ability to make IL-17A on a per cell basis is a ligand dependent process (**Fig.2A,B**).

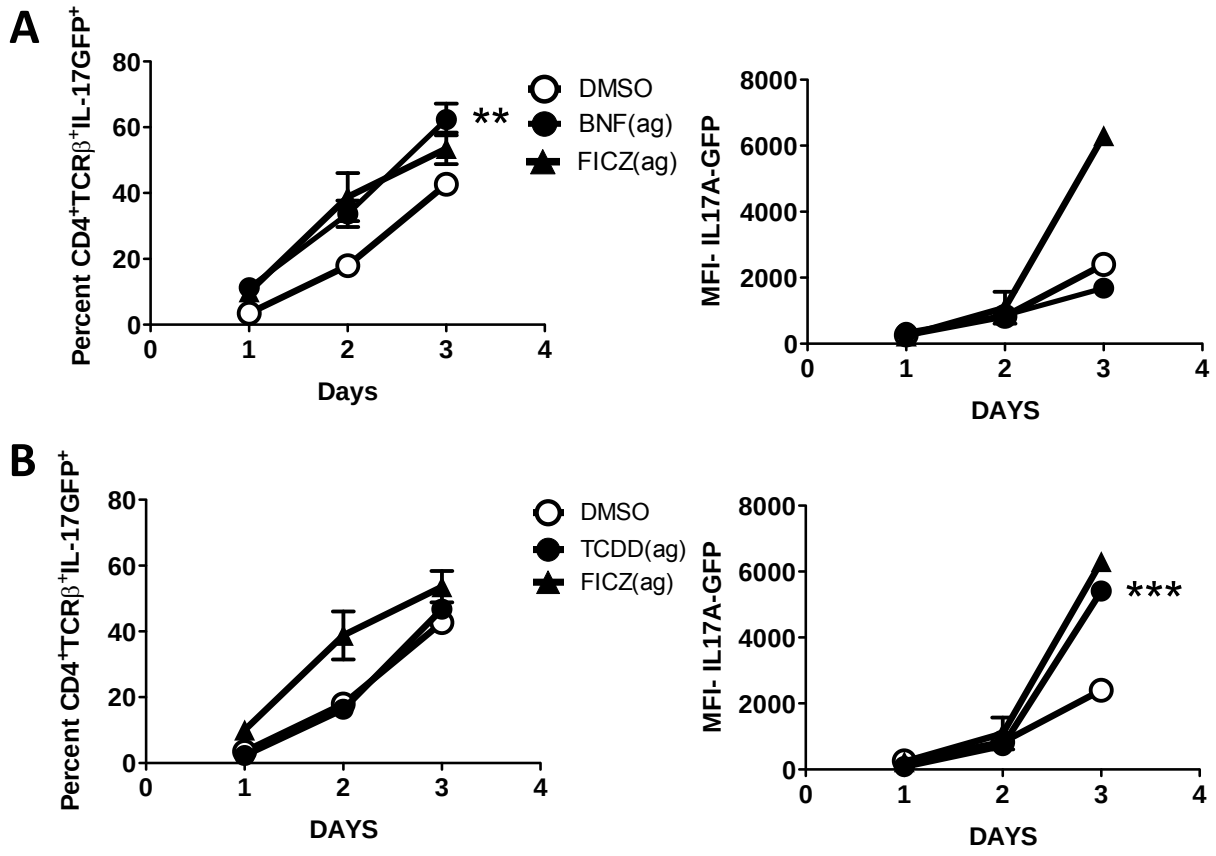


Fig. 2. AHR ligands influence Th17 differentiation via the DRE-driven pathway. Naïve $CD4^+$ T cells isolated from IL-17A-GFP reporter mice were cultured for 3 days with α-CD3/CD28 under Th17 skewing condition in the presence or absence 2 μM of β-naphthoflavone (β-NF) (A), 100 nM of 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) (B) or 200 nM of 6-Formylindolo (3,2-b) carbazole (FICZ). The cells were analyzed for the frequency of GFP^+ cells (left panel) and for the mean fluorescence intensity (MFI) of GFP (right panel) by on the indicated time points flow cytometry. Representative plot of atleast 3 independent experiments. Data represents Mean±SEM of n≥3 mice, *p<0.05 by 2-way ANOVA.

On the other hand, under similar conditions SGA-360 suppressed both frequency of Th17 cells and the MFI of IL-17A, which is in contrast to the complete antagonist GNF that only suppressed the frequency of Th17 cells. (**Fig.3 A & B**). These results suggest

that selectively inhibiting just the DRE mediated canonical AHR pathway can suppress the differentiation of naïve $CD4^+$ T cells into the Th17 lineage, indicating that regulation of IL-17A is mediated by DRE dependent pathway.

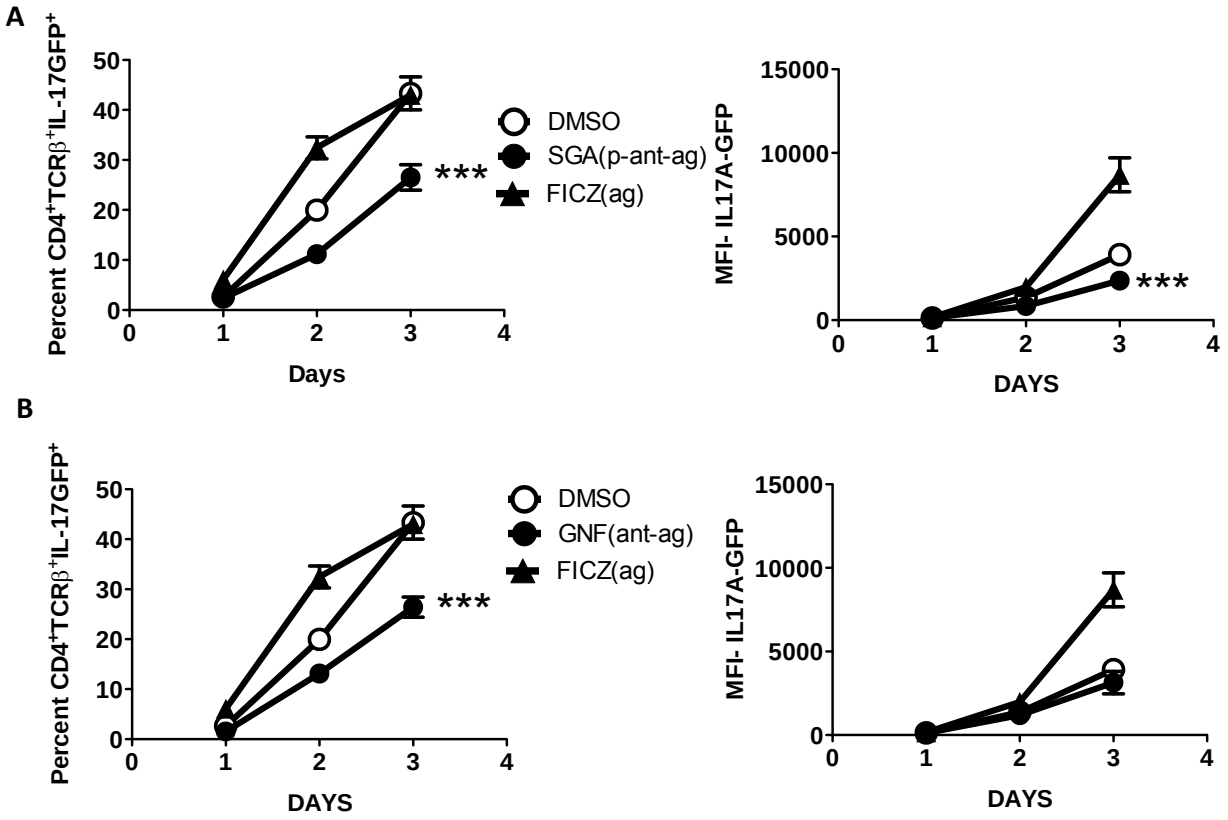
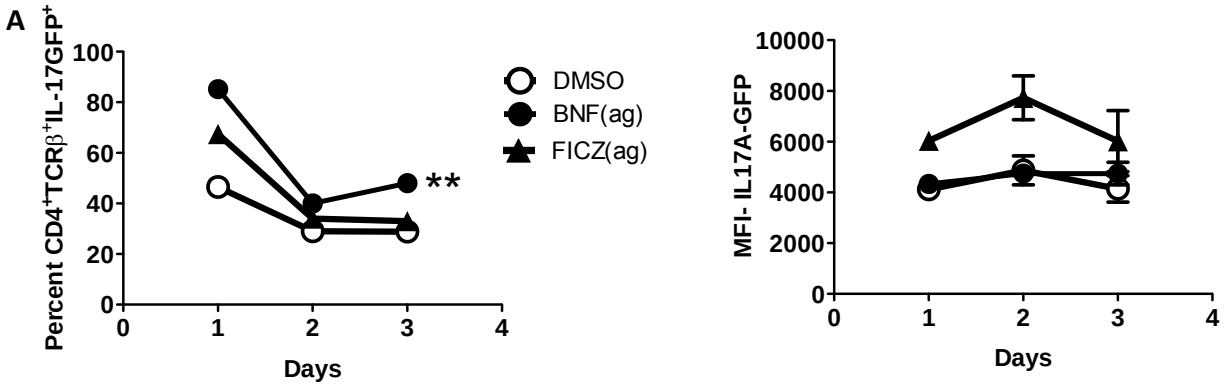


Fig. 3. AHR ligands influence Th17 differentiation via the DRE-driven pathway. Naïve $CD4^+$ T cells isolated from IL-17A-GFP reporter mice were cultured for 3 days with α-CD3/CD28 under Th17 skewing condition in the presence or absence 2 μM of SGA-360 (A), 100 nM of GNF (B) or 200 nM of 6-Formylindolo (3,2-b) carbazole (FICZ). The cells were analyzed for the frequency of GFP^+ cells (left panel) and for the mean fluorescence intensity (MFI) of GFP (right panel) on the indicated time points by flow cytometry. Representative plot of atleast 3 independent experiments. Data represents Mean±SEM of n≥3 mice, *p<0.05 by 2-way ANOVA.

We also analyzed the effects of these compounds on already differentiated Th17 cells *in vitro*. To this end, we added the compounds after differentiation of naïve $CD4^+$ T cells into Th17 cells and found that though β-NF could significantly increase the frequency of Th17 cells beyond that seen with FICZ, there was no difference in the amount of IL-17A produced on a per cell basis (**Fig.4 A**). On the other hand TCDD increased both the frequency IL-$17A^+$ cells and the MFI of IL-17A in already differentiated Th17 cells suggesting that the AHR agonists β-NF and TCDD differentially regulates IL-17A expression in Th17 versus naïve $CD4^+$ T cells. (**Fig.4 B**). Neither SGA-360 nor GNF could modulate the expression of IL-17A under these conditions (**Fig.4 C & D**). This suggests that, while agonists of AHR could further enhance IL-17A responses, inhibiting only the DRE-mediated activity of AHR or both the DRE and non-DRE activity does not affect the expression of IL-17A by already differentiated Th17 cells.



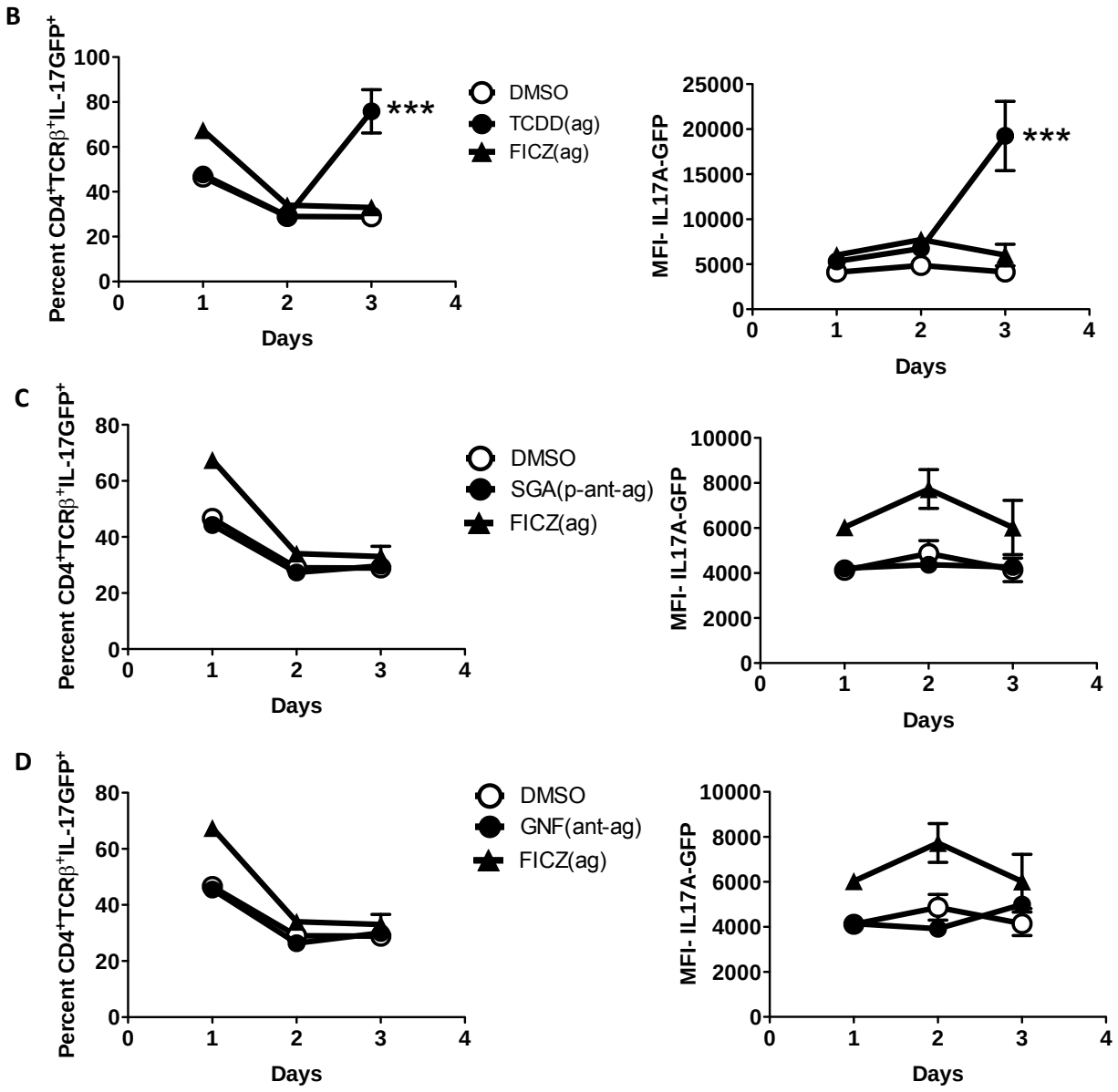


Fig. 4. AHR ligands influence Th17 differentiation via the DRE-driven pathway. Naïve $CD4^+$ T cells isolated from IL-17A-GFP reporter mice were cultured for 3 days with α-CD3/CD28 under Th17 skewing condition and subsequently restimulated under Th17 skewing condition in the presence or absence 2 μM of β-naphthoflavone (β-NF) (A), 100 nM of 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) (B), 2 μM of SGA-360 (C), 100 nM of GNF (D) or 200 nM of 6-Formylindolo (3,2-b) carbazole (FICZ) for 3 days. The cells were analyzed for the frequency of

GFP^+ cells (left panel) and for the mean fluorescence intensity (MFI) of GFP (right panel) on the indicated time points by flow cytometry. Representative plot of atleast 3 independent experiments. Data represents Mean±SEM of n≥3 mice, *p<0.05 by 2-way ANOVA.

2.2.3 Foxp3 expression is regulated by both DRE and non-DRE driven AHR activity under Treg polarizing condition

Similar to Th17 differentiation, AHR is also induced during Treg differentiation [6] . This prompted us to investigate the role of these compounds on the differentiation of naïve $CD4^+$ T cells into Tregs. We found that the AHR agonist behaved differently in affecting differentiation of Tregs as assessed by induction of Foxp3 expression by $CD4^+$ T cells. TCDD and FICZ both induced whereas β-NF had no effect on this process. Inhibiting only the DRE- driven activity by SGA-360 had no effect on the differentiation of Tregs but inhibiting both DRE and non-DRE mediated AHR activity with GNF inhibited Treg differentiation (**Fig.5**). This suggests that inhibition of the DRE- independent pathway can suppress induction of Foxp3 under Treg skewing conditions is mediated by inhibition of the DRE-independent pathway. It is interesting to note that even under Treg polarizing conditions β-NF and FICZ increased the frequency of the IL-$17A^+$ $CD4^+$ T cells (**Fig.6 A**)**.** Under these conditions TCDD, SGA-360 and GNF did not induce IL-17A. (**Fig.6 B, C & D**).

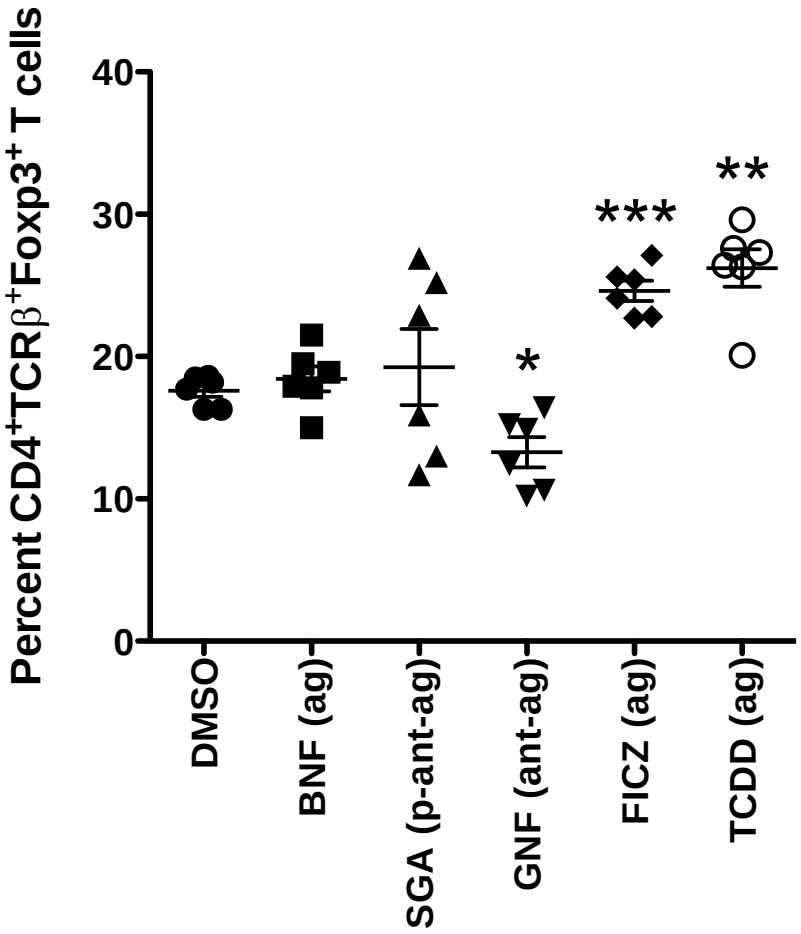
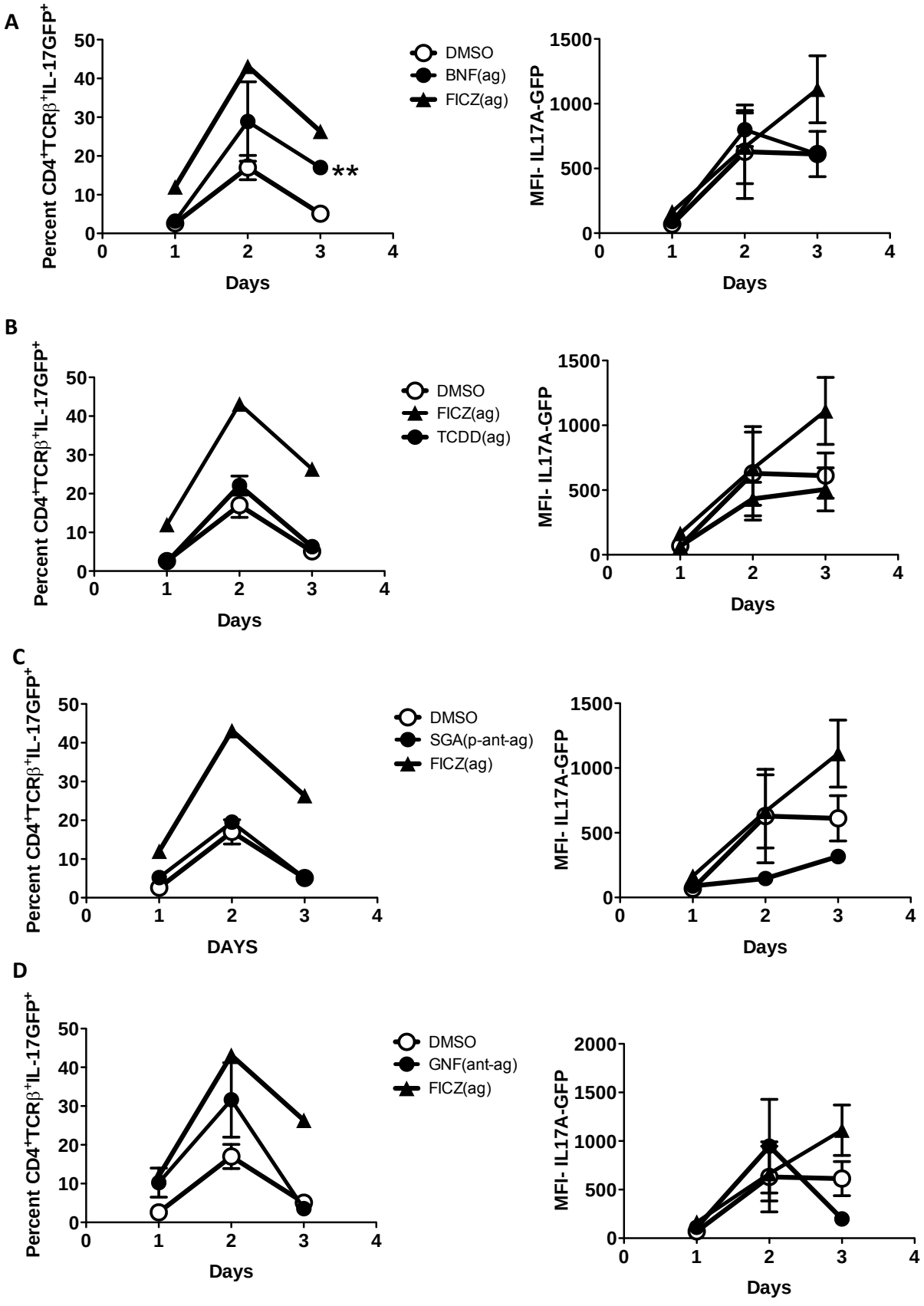


Fig. 5. Foxp3 expression is regulated by both DRE and non-DRE driven AHR activity under Treg polarizing condition. Naïve $CD4^+$ T cells isolated from IL-17A-GFP reporter mice were cultured for 3 days with α-CD3/CD28 under Treg skewing condition in the presence or absence 2 μM of β-naphthoflavone (β-NF), 100 nM of 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), 2 μM of SGA-360, 100 nM of GNF or 200 nM of 6-Formylindolo (3,2-b) carbazole (FICZ). The cells were analyzed for the frequency of $Foxp3^+$ cells by flow cytometry. Data represents Mean±SEM of 2 independent experiments with n≥3 mice per experiment, *p<0.05 by paired students *t* test.


A
Percent CD4+TCRβ+IL-17GFP+
Days
DMSO
BNF(ag)
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MFI- IL17A-GFP
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Percent CD4+TCRβ+IL-17GFP+
Days
DMSO
FlCZ(ag)
TCDD(ag)
MFI- IL17A-GFP
Days
C
Percent CD4+TCRβ+IL-17GFP+
DAYS
DMSO
SGA(p-ant-ag)
FlCZ(ag)
MFI- IL17A-GFP
Days
D
Percent CD4+TCRβ+IL-17GFP+
Days
DMSO
GNF(ant-ag)
FlCZ(ag)
MFI- IL17A-GFP
Days

Fig. 6. β-NF and FICZ promotes IL-17A expression under Treg polarizing condition. Naïve $CD4^+$ T cells isolated from IL-17A-GFP reporter mice were cultured for 3 days with α-CD3/CD28 under Treg skewing condition in the presence or absence 2 μM of β-naphthoflavone (β-NF) (A), 100 nM of 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) (B), 2 μM of SGA-360 (C), 100 nM of GNF (D) or 200 nM of 6-Formylindolo (3,2-b) carbazole (FICZ). The cells were analyzed for the frequency of GFP^+ cells (left panel) and for the mean fluorescence intensity (MFI) of GFP (right panel) on the indicated time points by flow cytometry. Representative plot of atleast 3 independent experiments. Data represents Mean±SEM of n≥3 mice, *$p<0.05$ by 2-way ANOVA.

2.3 Discussion

AHR has been shown to act as a sensor for environmental toxins such as dioxin, polycyclic halogenated compounds and structurally related compounds [24, 25]. It is becoming increasingly clear that AHR also plays an important role in the regulation of signaling in immune cells. Also, it is well known that environmental and dietary compounds that are AHR ligands play important roles in the induction of immune cell mediated inflammatory and autoimmune diseases and hence modulation of AHR is attractive as a strategy for control of these diseases [11]. Recent studies have identified AHR as having dual roles in the regulation of Th17 and Treg responses dependent on the ligand. It is known that Treg and Th17 cells have reciprocal relationship in their differentiation and AHR has been shown to be one of the common transcription factors that can regulate differentiation in the two cell populations [6]. In this study we have used three agonists TCDD, FICZ and β-NF to elucidate the effects of AHR activation on their effect on Th17/ Treg differentiation *in vitro*. Our findings indicate that AHR agonists have very different effects on the differentiation of Th17. FICZ, was able to enhance naïve $CD4^+$ T cell differentiation towards a Th17 phenotype and induce the expression of IL-17A, whereas β-NF could only enhance the frequency of Th17 cells. This shows that β-NF is able to drive a higher number of naïve $CD4^+$ T cells towards a Th17 phenotype but was unable enhance the production of IL-17A cytokine. By contrast, TCDD which is known to activate AHR to selectively upregulate FoxP3 in $CD4^+$ T cells under Treg polarizing conditions, was able to drive enhanced production of IL-17A cytokine on a per cell basis without increasing the frequency of Th17 cells under Th17 polarizing conditions.

Altogether these data suggest that AHR agonists may bind to distinct co-factors or differentially activate downstream signaling pathways, with resultant differences in the ability to regulate differentiation of Th17 cells and IL-17A production by Th17 cells. On the other hand, we found that both the complete antagonist GNF and the partial antagonist SGA-360 inhibited the differentiation of naïve $CD4^+$T cells into Th17 cells confirming the involvement of AHR activation in the process. We also examined the effects of these compounds on already differentiated Th17 cells and found that FICZ and TCDD could increase both the frequency and IL-17A expression from Th17 cells but β-NF only increases the frequency of Th17 cells which shows these agonists can further promote IL-17A secretion by terminally differentiated Th17 cells. Under the same conditions neither SGA-360 nor GNF had any effect on the frequency of $IL\text{-}17A^+$ cells as well as the secretion of IL-17A showing that DRE-mediated suppression is not effective on already lineage committed Th17 cells.

TCDD has been shown to have immunosuppressive effects by activating AHR and promoting Treg cell differentiation, via direct regulation of Foxp3 expression [6]. In this study we have identified the differential effects of the AHR ligands under T regulatory cell polarizing conditions. We found that the agonists TCDD and FICZ can promote Tregs from naïve $CD4^+$ T cells, whereas β-NF neither inhibited nor promoted the Treg cell differentiation. This indicates that β-NF may regulate AHR to modulate Foxp3 via a different mechanism than that of FICZ or TCDD even though all of them activate AHR through a DRE-mediated pathway. The partial antagonist SGA-360 had no effect on the differentiation of Tregs but the complete antagonist GNF suppressed it, suggesting that non-DRE mediated AHR activation may be able to suppress expression of Foxp3. In

other words, the regulation of Foxp3 expression by AHR can be mediated both by the DRE and non-DRE-driven responses dependent on the ligand. This suggests that AHR agonist and antagonist compounds can not only differentially regulate Th17 versus Treg differentiation but could also potentially influence the function of differentiated cells by controlling the frequency or the amount of IL-17A produced by these cells. These results further underscore the ligand specific functions of these compounds in the generation and/or function of Th17 and Treg cells. This is interesting because to our knowledge this is the first report that suggests a role for AHR signaling in the regulation of $CD4^+$ T cell plasticity.

Our results suggest that AHR activation can result in different outcomes in a ligand specific manner. It is known that AHR can interact with other transcription factors like cMaf, Stat1 to regulate different subsets of $CD4^+$T cells [26]. AHR is known to have crosstalk with receptors like estrogen and retinoic acid receptors, which may influence the dichotomous behavior of AHR in a ligand specific manner to modulate the Th17/Treg balance in a ligand specific manner. Thus ligand specific modulation of AHR makes it an attractive therapeutic target for the treatment autoimmune and chronic inflammatory diseases that have a strong correlation with environmental toxins and disregulation of Th17/Treg balance.

2.4 Materials and Methods

2.4.1 Mice and treatments. OTII and IL17A-GFP reporter mice were purchased from Jackson laboratories and Biocytogen respectively. All mice were used between 6- and 12-wk of age and maintained in a specific pathogen free environment. All experiments were performed in accordance with the regulations of the Office of Research Protection's Institutional Animal Care and Use Committee at Cornell University and the National Institutes of Health.

2.4.2 Cytokine analysis. OTII splenocytes and lymph node cells were cultured in IMDM media at 2million/ml concentration in triplicate and except the media control, all wells were added ovalbumin (Sigma) at 10ug/ml and compounds β-NF and SGA 360 at 5μM on the first day of the culture. 5 days later the supernatants were collected and the cytokines were quantified by Bioplex a Luminex system (Bio-Rad) with Milliplex plates (Millipore).

2.4.3 Quantitative PCR. RNA was extracted either using TRIZOL reagent (Invitrogen) or using the RNAeasy kit (Invitrogen) and cDNA was generated with a kit from You Prime First-Strand beads kit (GE Biosciences). q-PCR was then performed with all values normalized to respective GAPDH values and the data expressed as 2^{-DDCt} (where Ct is threshold cycle), the values are represented as fold change over respective controls.

2.4.4 T cell purification and polarizing conditions. Naïve T cells ($CD4^+TCR\beta^+CD44^{low}CD62L^{high}$) were isolated from spleens and lymph nodes of IL-17A reporter mice using Naïve $CD4^+$ T cell isolation kit from Miltenyi Biotech using manufacturers recommendations. Th17 polarizing conditions included 1 μg/mL α-murine CD3 and 3 μg/mL α-murine CD28 (BD Biosciences), 20 ng/mL IL6, 5 ng/mL TGFβ1 (Peprotech) and 10 μg/mL of α-IFN-γ. Treg polarizing conditions included 1 μg/mL α-murine CD3 and 3 μg/mL α-murine CD28 (BD Biosciences), 5ng/mL of TGF β1, 25 ng/mL of IL-2 (Peprotech) and 10 μg/mL of α-IFN-γ. Cells were cultured in IMDM media (Sigma).

2.4.5 Analysis of transcription factors and flow cytometry. Foxp3 fixation/ permeabilization kit (eBioscience) was used to fix/ permeabilize cells and then stained for the indicated surface proteins, and transcription factors. LSRII flow cytometer (BD Biosciences) was used to acquire data and the data was analyzed with FlowJo (TreeStar).

2.4.6 Data analysis. Statistical analysis was done with Student's *t* test and two-way ANOVA using GraphPad Prism version 5.00 for Windows (GraphPad, San Diego, CA). Differences with probability $p \leq 0.05$ were considered statistically significant.

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Chapter 3

Regulation of Th17 responses in acute and chronic inflammatory diseases by AHR ligands

3.1 Introduction

Th17 cells are a distinct subtype of $CD4^+$ T cells which secrete cytokines like IL-17A, IL-17F, IL-21, IL-22 and mediate autoimmune pathologies like experimental autoimmune encephalitis (EAE), collagen-induced arthritis (CIA) and psoriasis [1-3]. While Th17 cells have been well characterized for their pro-inflammatory role, they also play a protective role in the clearance of extracellular pathogens and fungi by promoting neutrophil recruitment and IgG mediated opsonization [4]. In the gut, depending on the cytokine environment, Th17 cells can mediate protective function by promoting gut barrier function and enhancing antimicrobial defense with the help of the co-expressed cytokine IL-22 [5, 6].

C. rodentium is a gram-negative bacteria which causes distal colitis in the large intestine of mice and has been used as an *in vivo* murine model to study protective Th17 responses. Infection is characterized by mucosal thickening and crypt hyperplasia due to epithelial cell proliferation. Upon oral infection, *C. rodentium* attaches to the epithelial cell surface (enterocytes) and induces the formation of the characteristic attachment/ effacement (A/E) lesion induced by enteropathogens that helps in effacement through the brush border microvilli and pedestal formation, which is very similar to enteropathogenic and enterohemorragic E. coli infection in humans [7, 8]. Colonic hyperplasia can be observed as early as 4 days post infection and the peak of the disease as assessed by bacterial burden is highest between days 10-12 post infection [7]. Both the epithelial cells that act as the first barrier to infection preventing trafficking of bacteria from the intestinal lumen and the adaptive immune system are critical for bacterial clearance [7]. In an immune-competent mouse, *C. rodentium* causes a

transient infection that is completely cleared by 14-21 days post infection with the induction of $CD4^+$ T cell responses mainly driven by Th17 cells. Thus Th17 cells play a critical role in the protection against *C. rodentium* infection by promoting bacterial clearance. AHR has been shown to play an important role in Th17 cell differentiation and we have recently shown that AHR can be modulated with agonist and antagonist compounds to regulate Th17 differentiation (refer to Chapter 2). Here we show that the addition of an AHR agonist, β-NF during *C. rodentium* infection enhanced protective Th17 responses and promoted bacterial clearance. On the other hand, administration of an AHR partial antagonist, SGA-360 resulted in lower Th17 responses and higher bacterial burden at the early stages of *C. rodentium* infection.

Hypersensitivity pneumonitis (also called extrinsic allergic alveolitis, EAA) is an inflammation of the alveoli within the lungs caused by hypersensitivity to aerosolized antigen. It is characterized by mononuclear infiltrates, collagen deposition and fibrosis in the lungs. Recent studies have shown Th17 driven processes mediates hypersensitivity pneumonitis [9, 10]. Using a chronic model of hypersensitivity pneumonitis induced by *Saccharopolyspora rectivirgula* (*S. rectivirgula*), we show that the agonist β-NF enhanced IL-17A production by fully differentiated Th17 cells. The partial antagonist SGA-360 and complete antagonist GNF on the other hand had minimal effect on IL-17A production but promoted anti-inflammatory $CD4^+$ T cell responses. This work has implications for better understanding of regulation of Th17 responses by AHR signaling.

3.2 Results

3.2.1 β-NF protects mice against *C. rodentium* infection

IL-17A produced by Th17 cells play a vital role in adaptive immune responses to protect against *C. rodentium* infection. We have shown in Chapter 2 that AHR agonist, β-NF, enhances the development of Th17 cells from naïve $CD4^+$ T cells. We therefore hypothesized that β-NF, would promote mucosal Th17 responses to attenuate disease severity by aiding in bacterial clearance. It has been previously shown that upon *C. rodentium* infection mice develop minor weight loss due to diarrhea, one of the clinical signs of disease. Vehicle treated CR infected mice showed consistent reduction in body weight starting from 1 day post infection (DPI). By contrast, β-NF treated mice maintained their body weight throughout the course of infection (**Fig1A**).

Upon infection, *C. rodentium* can be recovered in the fecal pellets of mice starting from 4 DPI, a process referred to as *bacterial shedding* [11, 12] . In order to evaluate if administration of β-NF influenced bacterial burden, we monitored bacterial shedding at various time points post *C. rodentium* infection in vehicle or β-NF treated mice. Vehicle treated mice showed a typical pattern of bacterial burden with ~10^9 CFU of *C. rodentium* recovered per gram of feces at 6DPI.The bacterial burden peaked at 10 DPI reaching a maximum of around 2x10^9 CFU and this was completely cleared by 14 DPI. In line with our previous results that β-NF treated mice had no body weight loss, we observed that the bacterial burden was significantly lower at both 6 and 10 DPI in these mice. Also, there was a significant delay in the peak of bacterial shedding in β-NF treated mice with the highest bacterial burden at 14DPI (**Fig1B**). This suggests that administration of β-

NF protects against *C. rodentium* infection by both delaying the peak and reducing bacterial burden in the large intestine.

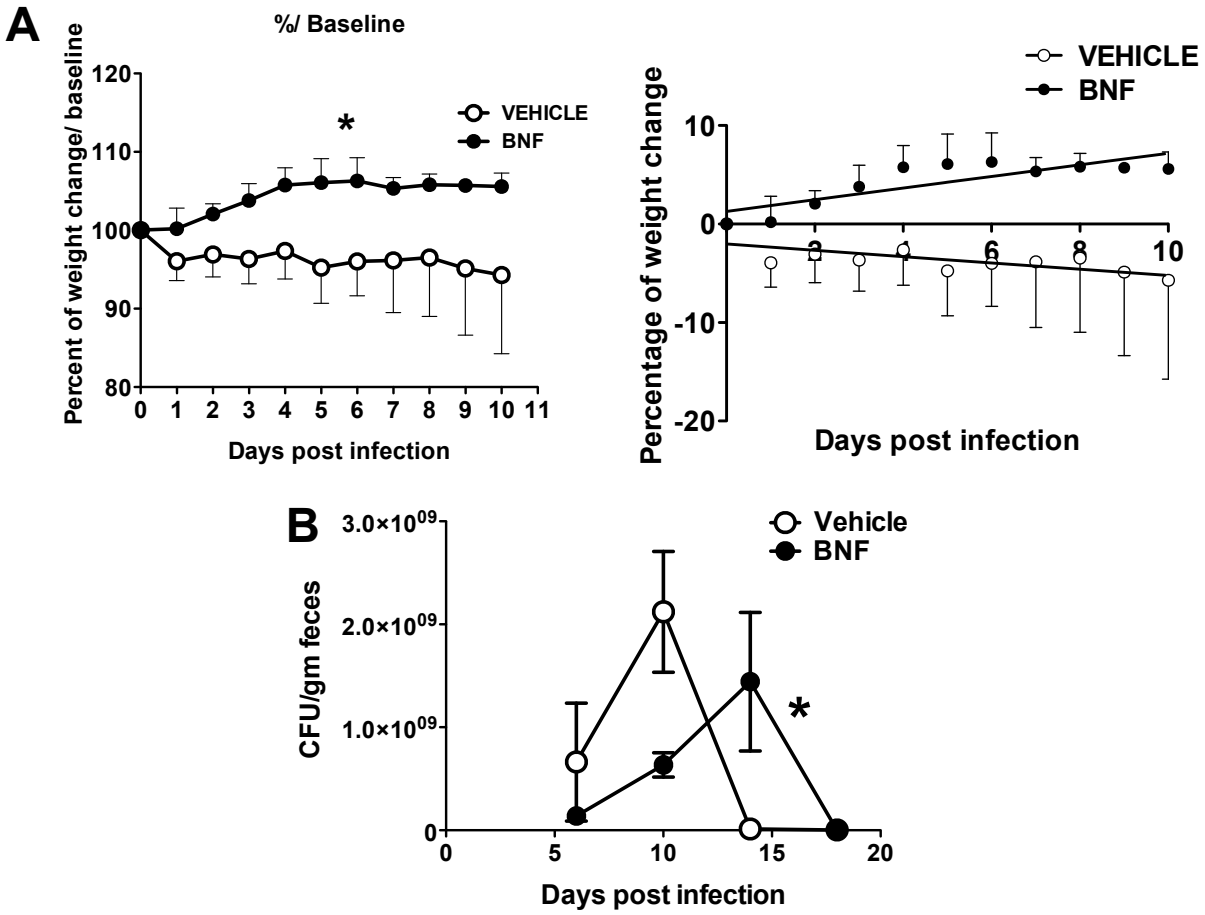

Fig. 1. **β-NF protects mice against *C. rodentium* infection**. A) IL17A-GFP reporter mice were infected with $5x10^9$ CFUs of *C. rodentium* on day 1 and treated with vehicle control or 50 mg/kg β-NF i.p. every other day starting from day 0 until the duration of the experiment and monitored for the change in body weight at the indicated time points represented as percent weight change (left panel) and linear regression analysis (right panel). B) Fecal pellets were collected from mice treated as above and analyzed for number of CFUs per gram of feces by plating dilutions on LB agar plates with Nalidixic acid. Representative plot of 3 independent experiments. Data represents Mean±SEM of n≥5 mice, *$p<0.05$ by 2 way ANOVA.

3.2.2 β-NF (agonist) promotes the differentiation of Th17 cells to protect against CR infection

It has also been shown that Th17 driven responses are the major contributor of adaptive immunity to *C. rodentium* infection [13].In chapter 2 we showed that agonist and antagonist ligands of AHR can selectively regulate both Th17 differentiation and/ or IL-17A production. To further confirm and extend these results *in vivo*, we infected IL-17A-GFP reporter mice with *C. rodentium* and assessed the differentiation of Th17 cells *in vivo* in the presence of the AHR modulating compounds. To this end, we analyzed a number of gut associated organs including the small and large intestinal lamina propria, intraepithelial lymphocyte compartment, gut draining mesenteric lymph nodes and the peyers patch. The use of this IL-17A reporter model gives us a unique opportunity to analyze the induction of Th17 responses *in vivo* without the need for ex vivo stimulation. In line with published reports we find that upon *C. rodentium* infection $TCR\beta^+$ T cells were the major source of $IL\text{-}17A^+$ GFP^+ cells (**Fig. 2A**) and the induction of Th17 responses to *C. rodentium* was the strongest in the large intestinal lamina propria, the primary site of infection 10 days post infection (**Fig. 2B & 2C**). This was further enhanced by the administration of β-NF (**Fig. 2B**), **suggesting** that the enhanced bacterial clearance in these mice is due to enhanced development of Th17 responses. We did not find a significant difference between the proportion of Th17 cells in the small intestinal lamina propria, intraepithelial lymphocyte compartment, gut draining mesenteric lymph nodes and the peyers patch in the vehicle versus β-NF treated mice suggesting that β-NF induces a strong local Th17 response to *C. rodentium* (**Fig. 2C**).

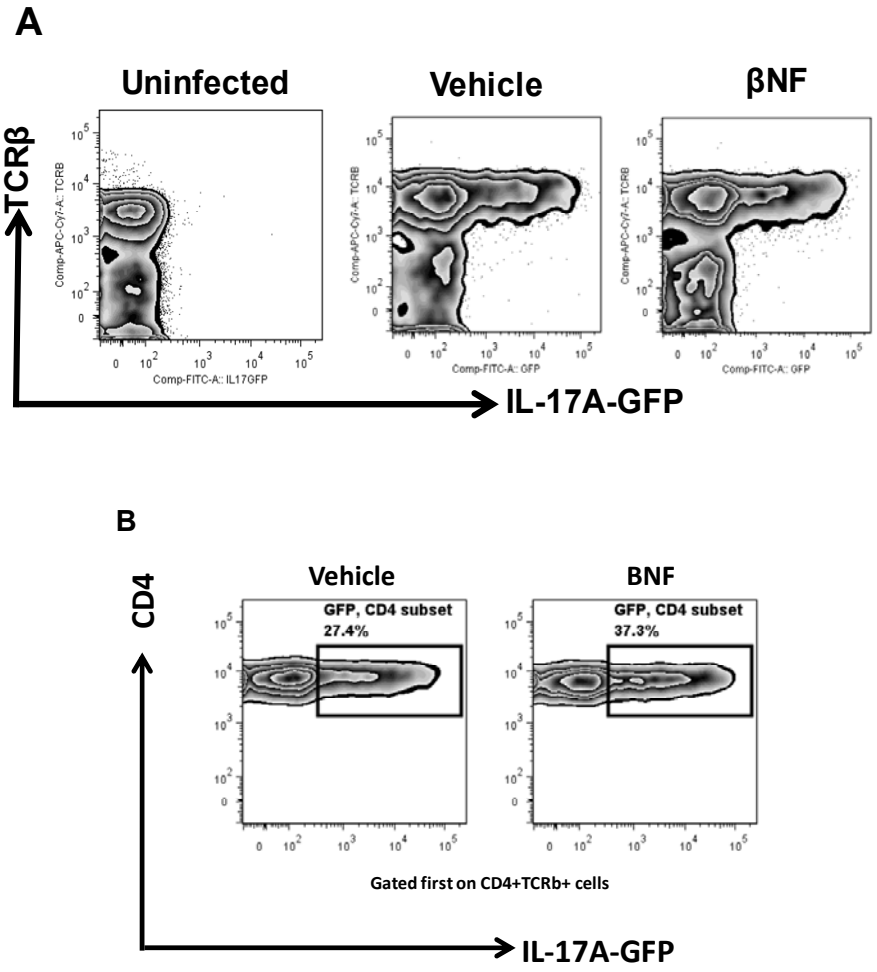

Fig. 2. **β-NF (agonist) promotes the differentiation of Th17 cells to protect against CR infection**. IL17A-GFP reporter mice were infected with 5*10^9 CFUs of *C. rodentium* on day 1 and treated with vehicle control or 50 mg/kg β-NF i.p. every other day starting from day 0 until the duration of the experiment and single cell suspensions obtained from large intestinal lamina propria (**A & B**), small intestinal lamina proria, intraepithelial lymphocytes, mesenteric lymph nodes and peyer's patch (**C**) were analyzed for the expression of IL-17A-GFP by TCRβ^+ (**A**) and CD4$^+$ TCRβ^+ cells (**B & C**). Representative flow plots shown in (**A & B**). Data represents Mean±SEM of n≥5 mice.

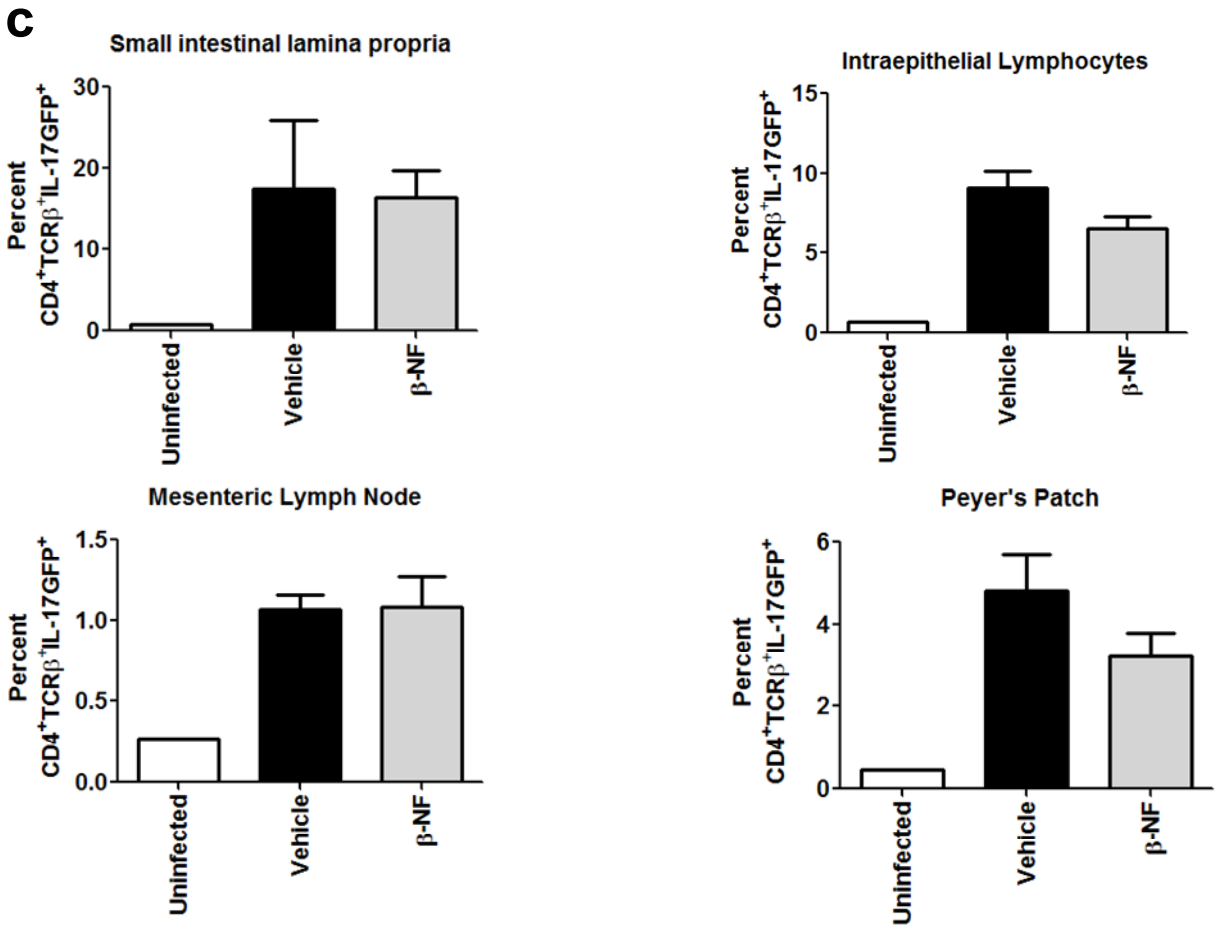

Fig. 2. **β-NF (agonist) promotes the differentiation of Th17 cells to protect against CR infection**. IL17A-GFP reporter mice were infected with 5*10^9 CFUs of *C. rodentium* on day 1 and treated with vehicle control or 50 mg/kg β-NF i.p. every other day starting from day 0 until the duration of the experiment and single cell suspensions obtained from large intestinal lamina propria (**A & B**), small intestinal lamina proria, intraepithelial lymphocytes, mesenteric lymph nodes and peyer's patch (**C**) were analyzed for the expression of IL-17A-GFP by $TCR\beta^+$ (**A**) and $CD4^+$ $TCR\beta^+$ cells (**B & C**). Representative flow plots shown in (**A & B**). Data represents Mean±SEM of n≥5 mice.

To further characterize the effect of β-NF on the generation of Th17 responses *in vivo* in the presence of β-NF we analyzed vehicle or β-NF treated *C. rodentium* infected mice at

various time points post infection. We find that β-NF treatment significantly enhanced Th17 responses to *C. rodentium* with the Th17 response highest at 10 DPI, the earliest time point we analyzed, as compared to vehicle treated controls that peaked at 14 DPI. There was no significant difference between the Th17 responses at 18 DPI between the vehicle and β-NF treated mice suggesting that while activation of AHR promotes differentiation of Th17 cells it might not influence Th17 responses at later stages when the bacteria has been completely cleared showing that β-NF only enhanced Th17 responses during an active immune reaction (**Fig. 3A**). These GFP^+ expressing $CD4^+$ T cells represented the population of effector Th17 cells generated in response to *C. rodentium*, as we obtained a similar percentage of IL17A producing $CD4^+$ T cells when we stimulated the large intestinal lamina propria cells isolated from mice at different stages of infection with PMA and Ionomycin ex vivo (**Fig. 3B**). We also found an inverse correlation between the magnitude of Th17 responses in the two groups and the abundance of bacteria in the fecal pellets (**Compare Fig. 1B and Fig. 3A**).Together these results suggest that activation of AHR by the agonist ligand β-NF promotes Th17 responses to protect against *C. rodentium* infection in mice.

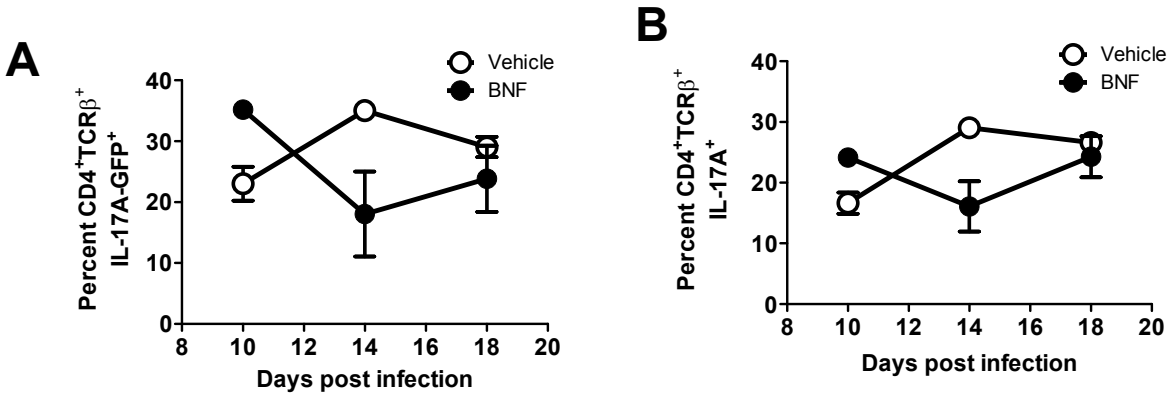

Fig. 3. **β-NF (agonist) promotes the differentiation of Th17 cells to protect against CR infection.** IL17A-GFP reporter mice were infected with $5x10^9$ CFUs of *C. rodentium* on day 1 and treated with vehicle control or 50 mg/kg β-NF i.p. every other day starting from day 0 until the duration of the experiment and single cell suspensions obtained from large intestinal lamina propria were analyzed for the proportion of $CD4^+$ $TCRb^+$ that express IL-17A-GFP at the indicated time points (**A**) or stimulated for 5 hours with PMA/ Ionomycin in the presence of Brefeldin A and stained for the level of intracellular IL-17A in $CD4^+$ $TCRb^+$ cells at the indicated time points (**B**). Representative plot of 3 independent experiments. Data represents Mean±SEM of n≥5 mice, *p<0.05 by 2 way ANOVA.

3.2.3 SGA-360 (partial antagonist) inhibits the differentiation of IL17A expressing Th17 cells during CR infection

To determine whether inhibition of AHR signaling influenced generation of effector Th17 cells in response to *C. rodentium* we compared bacterial burden and the induction of Th17 responses in the presence or absence of SGA-360.We find that, administration of SGA-360 resulted in a significantly higher bacterial burden early during CR infection (6 DPI), probably as a result of a suboptimal Th17 response (**Fig. 4A**). Since we saw maximal Th17 responses at 10 DPI a time frame that coincides with the highest bacterial burden, we decided to analyze vehicle and SGA-360 treated *C. rodentium*

infected mice at 10 DPI for Th17 responses. In line with increased bacterial burden we find that there was a significant reduction in the proportion of Th17 cells in the large intestinal lamina propria upon treatment with SGA-360 (**Fig. 4B**). These results suggest that *in vivo* differentiation of Th17 cells can be inhibited by the inhibition of AHR with resultant increase in bacterial burden.

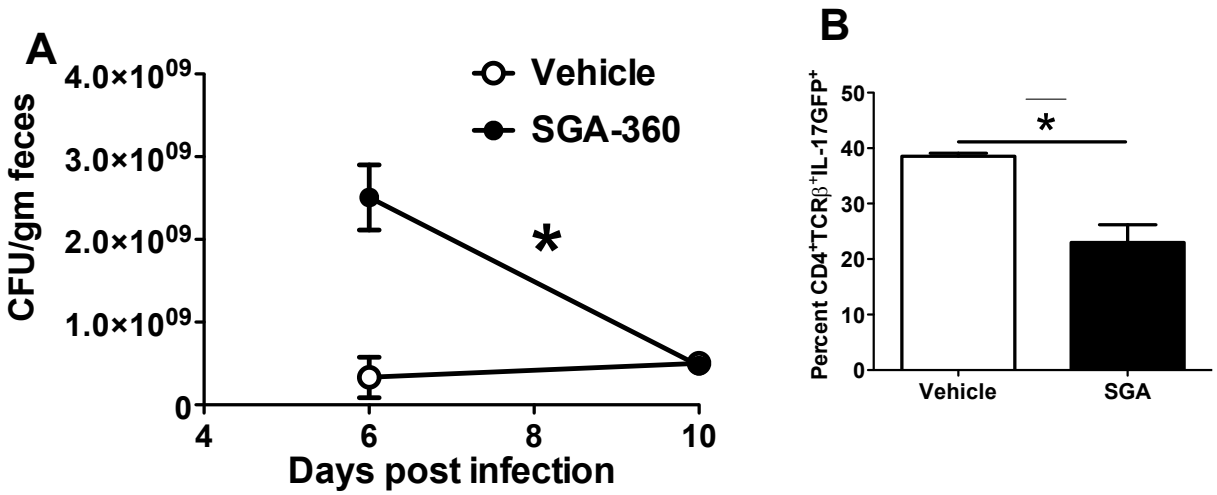

Fig. 4. **SGA-360 (partial antagonist) inhibits the differentiation of IL17A expressing Th17 cells during CR infection**. A) IL17A-GFP reporter mice were infected with $5x10^9$ CFUs of *C. rodentium* on day 1 and treated with vehicle control or 20 mg/kg SGA-360 i.p. every other day starting from day 0 until the duration of the experiment and the fecal pellets obtained at the indicated time points were analyzed for the number of CFUs per gram of feces. B) Single cell suspensions of large intestinal lamina propria cells from mice treated as above was analyzed for the proportion of $CD4^+$ $TCRb^+$ that express IL-17A-GFP at 10 DPI. Data represents Mean±SEM of n≥5 mice, *p<0.05 by 2 way ANOVA (A) and unpaired student t test (B).

3.2.4 Activation of AHR affects both the differentiation of Th1 and iTreg cells *in vivo*

AHR can bind Stat1 and prevent it from binding to IL-17A promoter and hence promoting Th17 differentiation [14] . It has also been shown that IFN-γ, the lineage defining cytokine of Th1 cells, can negatively regulate the differentiation of Th17 cells both in vitro and *in vivo* [2, 3, 13] . We next wanted to determine if modulation of AHR affected IFN-γ production by $CD4^+$ T cells *in vivo* in response to *C. rodentium*. To do this, we isolated large intestinal lamina propria cells from day 10 *C. rodentium* infected mice that were either treated with vehicle, β-NF or SGA-360. We find that, administration of β-NF resulted in a significant reduction in the proportion of large intestinal lamina propria $CD4^+$ T cells that were able to secrete IFN-γ but not the IFN-γ^+IL17A$^+$ double positive cells (**Fig. 5A**). SGA-360 on the other hand had minimal effect both on the differentiation of Th1 cells and the proportion of IFN-γ^+IL-17A$^+$ double positive cells (**Fig. 5B**). Thus addition of β-NF promotes Th17 differentiation either directly by actively repressing the differentiation of Th1 cells or indirectly by promoting Th17 differentiation at the expense of Th1 differentiation.

Foxp3$^+$ Treg cells play a critical role in immune cell homeostasis by controlling over-exuberant inflammatory responses. It has been previously shown that, AHR activation can promote the differentiation of iTregs in the intestinal mucosa by promoting the differentiation of tolerogenic dendritic cells in a retinoic acid dependant manner [15].Based on this, we wanted to determine if modulation of AHR with β-NF or SGA-360 affected the differentiation of iTregs in response to *C. rodentium*. We find that, while

SGA-360 had minimal effect, β-NF significantly enhanced the proportion of iTregs in the large intestinal lamina propria of *C. rodentium* infected mice (**Fig. 5C**).

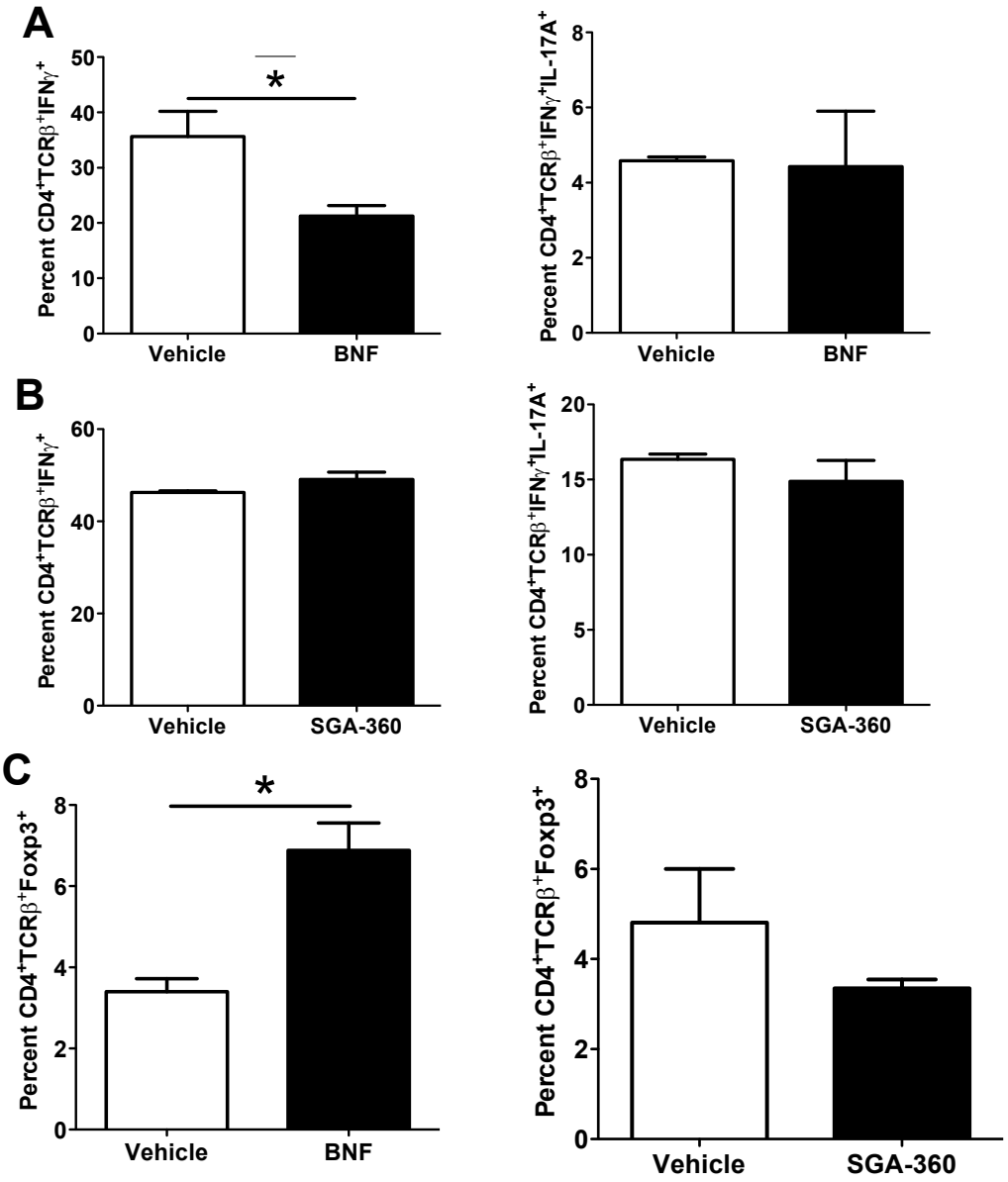

Fig. 5. **Activation of AHR affects both the differentiation of Th1 and iTreg cells *in vivo***. (A-C) IL17A-GFP reporter mice were infected with $5x10^9$ CFUs of *C. rodentium* on day 1 and treated with vehicle control or 50 mg/kg β-NF or 20 mg/kg SGA-360 i.p. every other day

starting from day 0 until the duration of the experiment and single cell suspensions of large intestinal lamina propria cells was analyzed for the indicated cell populations at 10 DPI. Data represents Mean±SEM of n≥5 mice, *p<0.05 by and unpaired student t test.

3.2.5 Modulation of AHR activity influences the secretion of IL-17A by Innate lymphoid cells

Innate lymphoid cells (ILCs) represent a class of recently identified innate immune cells that are developmentally closely related to T helper cells [16]. Group 3 ILCs are a subtype of ILCs that are similar to Th17 cells in their transcriptional and cytokine profile. Recently it has been shown that AHR plays an important role in the maintenance of steady-state levels of group 3 ILCs in the intestinal lamina propria [17] [18]. Although AHR is critical for the differentiation of Th17 cells, in the absence of AHR there is reduced IL-22 that results in increased colonization by the commensal segmented filamentous bacteria (SFB), which induces IL-17A secretion by both Th17 cells and ILCs [18-21]. Based on this, we wanted to determine how modulation of AHR activity using β-NF or SGA-360 would affect the differentiation of group 3 ILCs that can secrete IL-17A *in vivo* using the *C. rodentium* infection model. We analyzed the large intestinal lamina propria of vehicle, β-NF or SGA-360 treated *C. rodentium* infected mice at 10 DPI for the proportion of IL-17A$^+$ ILCs. We find that, activation of AHR with β-NF significantly increased the percentage of IL-17A$^+$ ILCs while SGA-360 inhibited AHR activity to inhibit differentiation of IL-17A$^+$ ILCs (**Fig. 6**). These results suggest that although at steady-state AHR signaling suppresses the differentiation of IL-17A$^+$ ILCs (through the AHR-IL-22 axis), inhibition of AHR results in reduced differentiation of IL-17A$^+$ ILCs in

response to *C. rodentium*. Thus targeting AHR is an attractive therapeutic strategy for modulation of IL-17A production by both innate and adaptive immune cells *in vivo*.

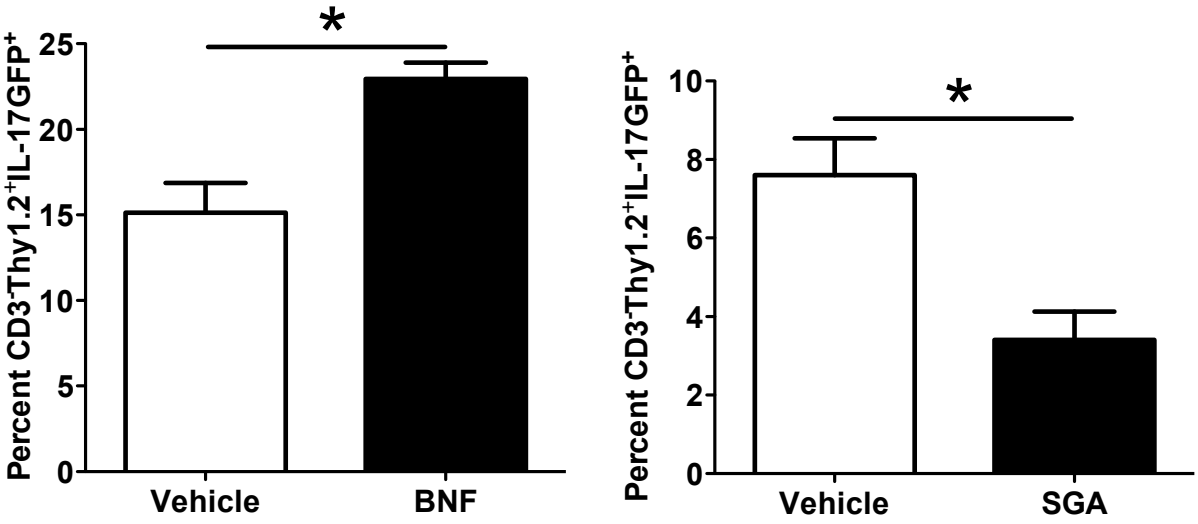

Fig. 6. **Modulation of AHR activity influences the secretion of IL-17A by Innate lymphoid cells**. IL17A-GFP reporter mice were infected with $5x10^9$ CFUs of *C. rodentium* on day 1 and treated with vehicle control or 50 mg/kg β-NF or 20 mg/kg SGA-360 i.p. every other day starting from day 0 until the duration of the experiment and single cell suspensions of large intestinal lamina propria cells was analyzed for the indicated cell populations at 10 DPI. Data represents Mean±SEM of n≥5 mice, *p<0.05 by and unpaired student t test.

3.2.6 Activation of AHR enhances IL-17A expression by differentiated Th17 cells *in vivo*

The above results suggest that modulation of AHR activity can differentially regulate Th17 differentiation *in vivo*. Next we wanted to determine if AHR signaling regulates the production of IL-17A by fully differentiated Th17 cells. To test this, we used a chronic Th17 mediated disease model, hypersensitivity pneumonitis that is induced by the gram-positive bacteria *S. rectivirgula*. Repeated challenge with *S. rectivirgula* induces chronic lung inflammation resulting in extrinsic allergic alveolitis or hypersensitivity pneumonitis that is characterized by the collagen deposition and fibrosis in the lungs [9]. Th17 cells that are induced in response to *S. rectivirgula* potentiate inflammation during the chronic stages of the disease and hence are pathogenic in this disease model. Based on this, we wanted to determine if modulation of AHR activity after disease development would influence Th17 responses. To address this, we intra nasally challenged IL-17A GFP reporter mice with *S. rectivirgula* for 3 consecutive weeks (3 times/week) and subsequently treated the mice intranasally with vehicle or AHR modulating compounds for 5 consecutive days on the 3rd week along with *S. rectivirgula* challenge. Administration of β-NF during the chronic stages of the disease, after induction of Th17 responses against *S. rectivirgula*, not only increased inflammation within the lungs with increased accumulation of $CD4^+$ T cells (**Fig. 7A**) but it also enhanced the production of IL-17A by differentiated Th17 cells (**Fig. 7B**). These results suggest that β-NF can enhance the production of IL-17A by fully differentiated Th17 cells *in vivo*.

IL-17A$^+$ γδ T cells have also been shown to mediate pathology during challenge with *S. rectivirgula* and γδ T cells express AHR. So we wanted to determine if the proportion of IL-17A$^+$γδ T cells was affected by addition of β-NF. Although β-NF did not enhance IL-17A production by γδ T cells, it promoted increased accumulation of γδ T cells in the lungs (**Fig. 7C & 7D**). The failure to enhance IL-17A production could be due to intrinsic differences in the requirement of AHR signaling by CD4$^+$ T cells versus γδ T cells or it could be secondary to the fact that γδ T cells have already reached their maximal threshold for IL-17A production in response to *S. rectivirgula* challenge (~70-80% of γδ T cells in vehicle treated *S. rectivirgula* challenged mice can make IL-17A).

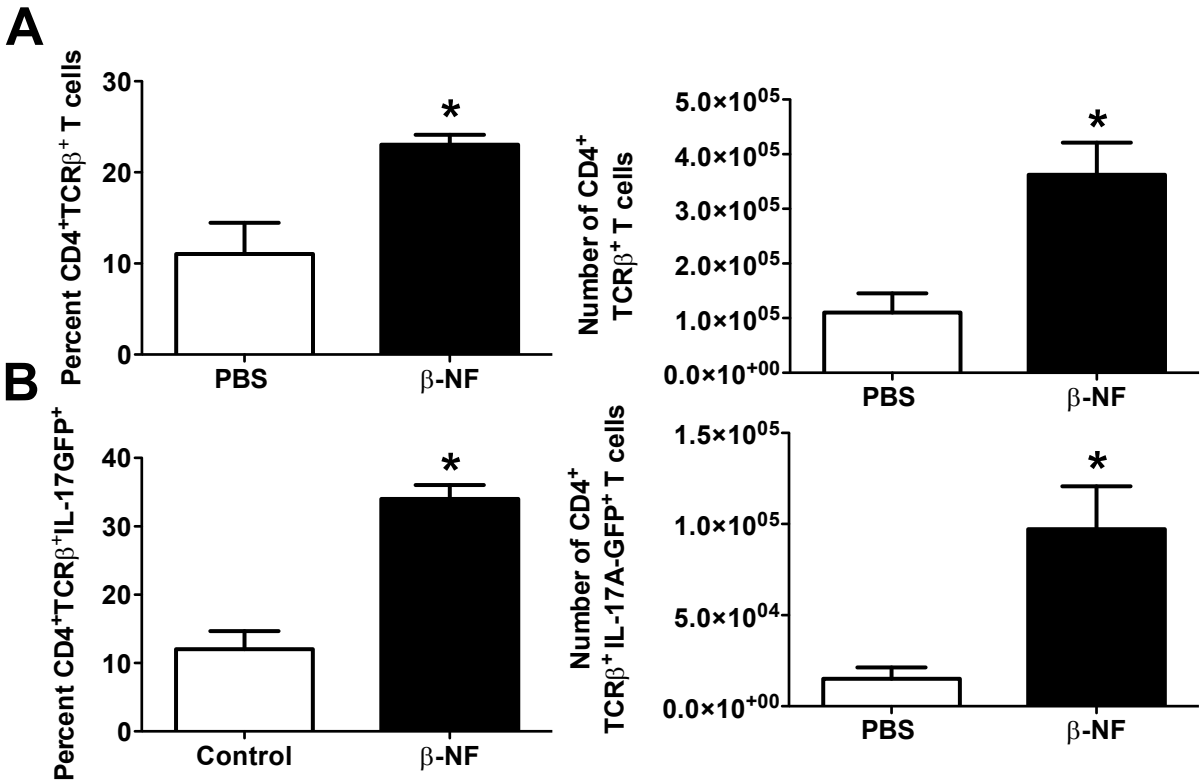

Fig. 7. **Activation of AHR enhances IL-17A expression by differentiated Th17 cells *in vivo***. A-D) IL17A-GFP reporter mice were challenged intranasally with *S. rectivirgula* (150mg/ mouse) for 3 consecutive days for 3 weeks followed treatment with either PBS or 0.4 mg/ mouse β-NF intranasally for 5 consecutive days on the 3rd week. Single cell suspension of lungs were stained and analyzed for the indicated cell populations. Data plotted both as percentage cells (left panels) and total cell number (right panels). Data represents Mean±SEM of n≥5 mice, *p<0.05 by and unpaired student t test.

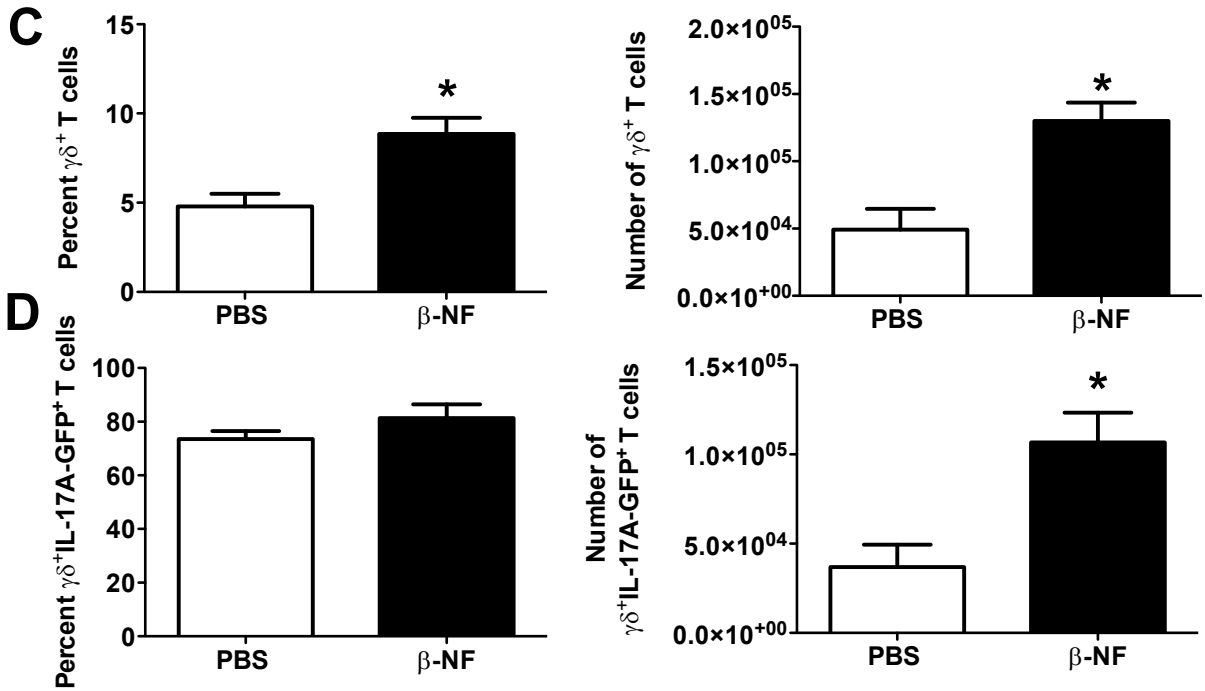

Fig. 7. **Activation of AHR enhances IL-17A expression by differentiated Th17 cells *in vivo***. A-D) IL17A-GFP reporter mice were challenged intranasally with *S. rectivirgula* (150mg/ mouse) for 3 consecutive days for 3 weeks followed treatment with either PBS or 0.4 mg/ mouse β-NF intranasally for 5 consecutive days on the 3rd week. Single cell suspension of lungs were stained and analyzed for the indicated cell populations. Data plotted both as percentage cells (left panels) and total cell number (right panels). Data represents Mean±SEM of n≥5 mice, *p<0.05 by and unpaired student t test.

We next wanted to determine if inhibition of AHR activity with the partial antagonist, SGA-360 or the complete antagonist GNF can influence production of IL-17A by differentiated Th17 cells. Intranasal administration of both SGA-360 and GNF inhibited the induction of CYP1a1 transcription in the lungs suggesting that both these compounds inhibited AHR activity (**Fig. 8A**). While addition of SGA-360 and GNF did not affect the levels of IL-17A transcripts in the lungs, there was a considerable reduction in IL-17F transcripts in both the treatment groups compared to vehicle controls (**Fig. 8B**). Interestingly, both SGA-360 and GNF treatment enhanced the expression of IL-4 and IFN-γ (**Fig. 8C**) suggesting that these partial and complete antagonist compounds can switch the cytokine profile in the lungs of *S. rectivirgula* challenged mice. In line with this, we did not see a significant change in the proportion of $CD4^+$ T cells or a reduction in the proportion of Th17 cells in the lungs of compound versus vehicle treated *S. rectivirgula* challenged mice (**Fig. 8D**) but there was a significant increase in the proportion of $CD4^+$ T cells that could make IL-4 (**Fig. 8E**). We did not find an increase in the percentage of IFN-γ^+ $CD4^+$ T cells in the lungs suggesting that the increase in IFN-γ transcripts we observed was not a result of increased accumulation of Th1 cells in the lungs (**Fig. 8F**). Addition of GNF also promoted the differentiation $Foxp3^+$ Treg cells in the lungs (**Fig. 8G**). Taken together, these results suggest that although SGA-360 and GNF cannot influence the secretion of IL-17A by fully differentiated Th17 cells they could promote anti-inflammatory $CD4^+$ T cell responses. It would be interesting to see if this switch in cytokine profile in the SGA-360 and GNF treatment groups influenced pathophysiology in the chronic stage of the disease.

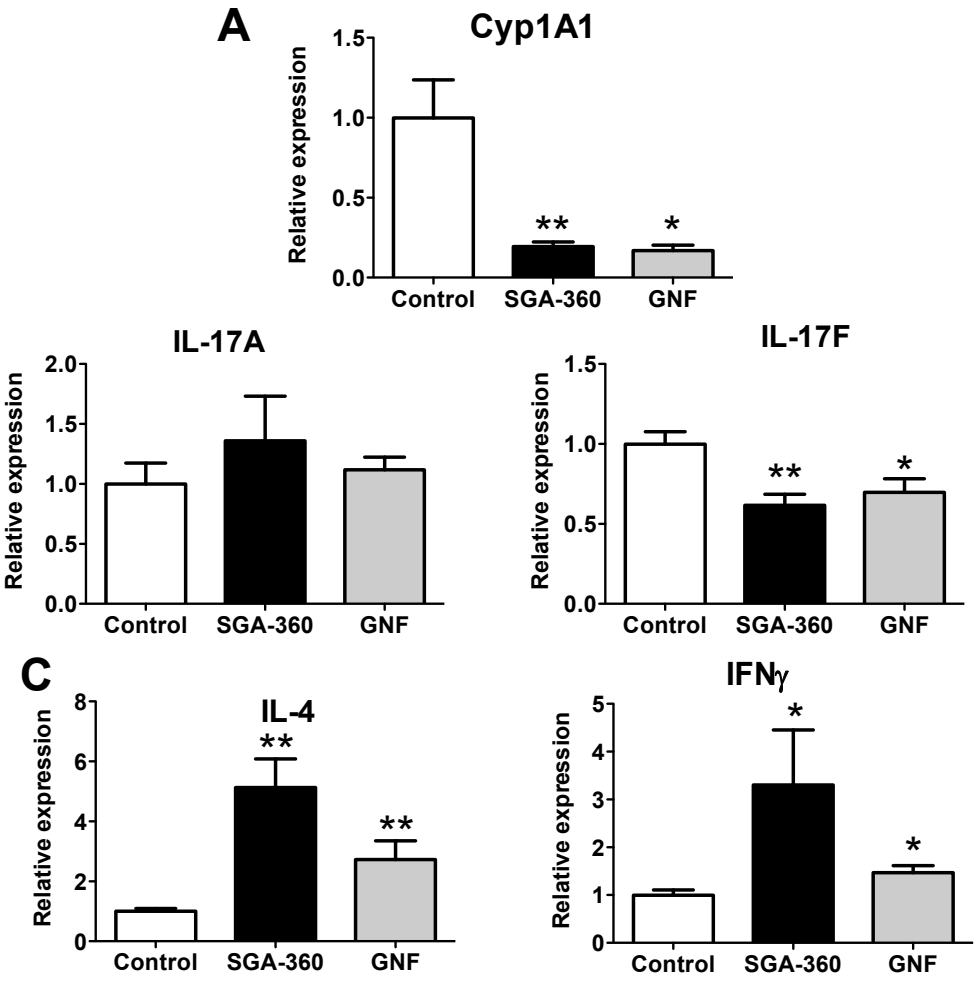

Fig. 8. Activation of AHR enhances IL-17A expression by differentiated Th17 cells *in vivo*. C57Bl/6 mice were challenged intranasally with *S. rectivirgula* (150mg/ mouse) for 3 consecutive days for 3 weeks followed treatment with either PBS or 0.4 mg/ mouse SGA-360 or GNF intranasally for 5 consecutive days on the 3rd week. A-C) qPCR analysis of indicated genes in the RNA isolated from lung tissue of mice treated as above, values are normalized to GAPDH. D-G) Single cell suspension of lungs isolated from mice treated as above were stained and analyzed for the indicated cell populations. Data plotted both as percentage cells except upper right panel which represents total cell number. Data represents Mean±SEM of n≥5 mice, *p<0.05 by and unpaired student's t test.

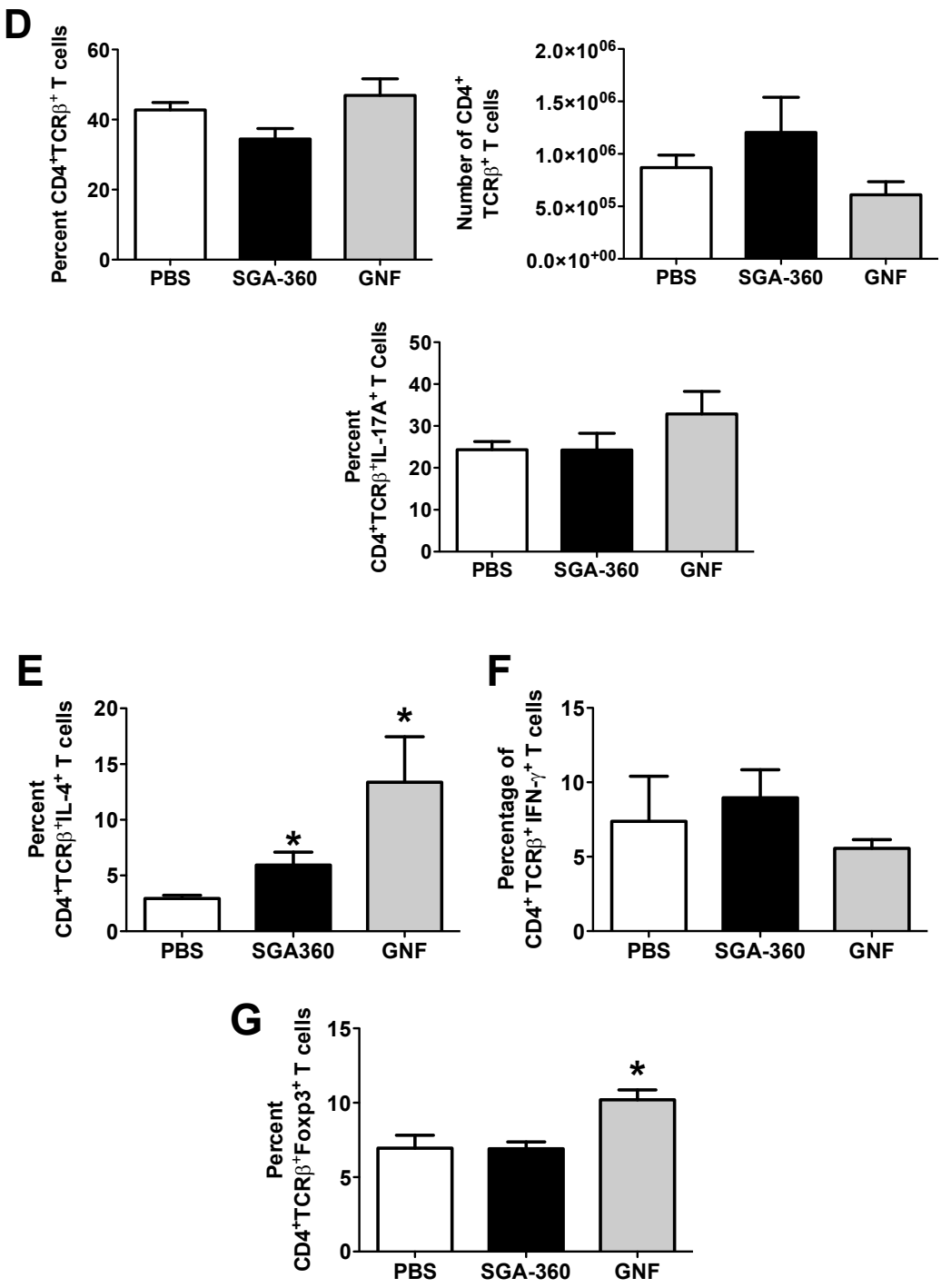

Fig. 8. Activation of AHR enhances IL-17A expression by differentiated Th17 cells *in vivo*. C57Bl/6 mice were challenged intranasally with *S. rectivirgula* (150mg/ mouse) for 3 consecutive days for 3 weeks followed treatment with either PBS or 0.4 mg/ mouse SGA-360 or GNF intranasally for 5 consecutive days on the 3rd week. A-C) qPCR analysis of indicated

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3.3 Discussion

AHR is expressed in both innate and adaptive immune cells and recently its role in modulating Th17/Treg balance in a ligand specific manner has opened up new avenues for therapeutics in autoimmune and allergic diseases. Activation of AHR in $CD4^+$ T cells exacerbates EAE by generation of pro-inflammatory Th17 cells in a ligand specific manner [22]. However, Th17 cells also have been shown to have protective function, for instance in the gut, and hence AHR activation in different disease models can have very different outcomes. In this study we used an intestinal bacterial infection model with *C. rodentium* in which Th17 cells are protective. and also a lung Hypersensitivity Pneumonitis model with *S. rectivirgula* where Th17 cells is pathogenic to assess the role of AHR signaling in regulation of Th17 responses *in vivo*.

Recently, a novel role of AHR in gut has been elucidated where AHR expression in innate immune cells called group 3 innate lymphoid cells (ILCs) are shown to maintain gut homeostasis by suppressing pathogenic Th17 cells in steady-state physiological conditions [18]. It was shown that AHR expression in group 3 ILCs regulate the AHR-IL-22 axis in restricting the aberrant expansion of commensal segmented filamentous bacteria (SFB) which increases Th17 population in the gut, thereby negatively regulating mucosal Th17 responses [18, 21]. We have shown that systemic activation of AHR by an agonist, β-NF during *C. rodentium* infection increased Th17 responses, which resulted in better clearance of bacteria as well as delay of the peak of infection. Even though there was an increase of IL-17A from $CD3^-Thy1.2^+$ ILCs with β-NF, the majority of IL-17A producers were T-cells, and β-NF specifically increased the IL-17A expression in large intestinal lamina propria, the site of bacterial infection.

It has been shown that IFNγ acts as a negative regulator of Th17 development and here we show that β-NF treated mice had less IFNγ producing $CD4^+$T cells, which could be one mechanism by which β-NF augments Th17 responses in *C. rodentium* infected mice [2, 3]. Apart from IL-17A, Th17 cells can also co-express IFNγ, which are regarded as pathogenic forms of Th17 responses and are responsible for worsening of encephalomyelitis, a mouse model of multiple sclerosis [23]. We found that administration of β-NF did not affect the $CD4^+$IL-$17A^+$$IFN\gamma^+$cells, suggesting that AHR activation via β-NF specifically enhances $CD4^+$IL-$17A^+$ cells which render a protective function through an augmented clearance of *C. rodentium*. Treatment with partial antagonist SGA-360 increased bacterial burden during early stages indicating a defective Th17 response due to inactivation of AHR via DRE-driven pathway. IL-17A expression was inhibited in SGA-360 vs vehicle treated mice at later time point even though there was no appreciable difference in bacterial burden. Since, SGA-360 only partly suppresses AHR activation through DRE, it is plausible that AHR activation via non-canonical protein-protein interaction may still induce other cytokines like IL-22 which can aid in bacterial clearance at later time points during *C. rodentium* infection [5, 18]. However, it is interesting to note that both β-NF and SGA-360 enhanced or suppressed IL-17A in ILCs respectively in large intestinal lamina propria, underscoring the specificity of these compounds to modulate Th17 response to *C. rodentium*.

It has been shown that fully differentiated Th17 cells generated *in vivo* retain AHR expression and hence it is conceivable that modulation of AHR activity in these cells can affect IL-17A production [24] . Similar to the acute *C. rodentium* infection model, in a chronic model of Th17 mediated disease, hypersensitivity pneumonitis induced by *S.*

rectivergula, β-NF enhanced Th17 responses. However, while the AHR antagonists SGA-360 and GNF had minimal effect on already established Th17 responses, they were able to enhance IL-4 production by $CD4^+$ T cells. In addition to this GNF promoted differentiation of Treg cells. Thus while activation of AHR in fully differentiated Th17 cells can further enhance IL-17A production, inhibition of AHR activity does not influence IL-17A production but can promote an anti-inflammatory environment. Fate mapping studies have shown that under chronic inflammatory settings like EAE, Th17 cells are more plastic and can be induced to secrete other cytokines to adopt alternate fates [24]. Also, it has been shown that AHR can affect both Th17 and Treg differentiation in a ligand specific manner [15, 22, 25] .Hence, it would be interesting to see if the $CD4^+$ T cells that were induced make IL-4 or express Foxp3 upon addition of AHR antagonists were IL-17A secreting Th17 cells prior to addition of the compounds. If true, this would imply that modulation of AHR could alter Th17 cell plasticity *in vivo* and hence offers attractive therapeutic opportunities for chronic inflammatory diseases.

3.4 Materials and Method

3.4.1 Mice and treatments. C57bl/6 and IL17A-GFP reporter mice were purchased from Jackson laboratories and Biocytogen respectively. All mice were used between 6- and 12-wk of age and maintained in a specific pathogen free environment.

For *C. rodentium* infection, Citrobacter rodentium strain ICC 169 the Nalidixic Acid resistant strain was cultured in LB with nalidixic acid at 50µg/ml over night and then mice were gavaged with $5x10^9$ CFUs (calculated based on OD) of *C. rodentium.* Bacterial shedding was calculated using the following formula

(Average # colonies (CFU) / 0.025 ml (volume plated)) x dilution factor = CFU/ml

Then factor in the initial addition of PBS to feces for homogenization

CFU / ml x 1 ml PBS / 0.1 g feces = CFU/ g feces

For hypersentivity pneumonitis experiments B6 mice were given 150µg (50µl) of *S. rectivirgula* (American Type Tissue Collection catalogue no. 29034) or sterile 1X PBS. Mice were lightly anesthetized with isoflurane and either *S.rectivirgula* or sterile PBS. was administered intranasally for 3 consecutive days for 3 weeks. *S. rectivirgula* was prepared by growing the microorganism in tryptic soy broth at 55°C with constant agitation. The *S. rectivirgula* culture was centrifuged, resuspended in sterile PBS, and the concentration of the SR protein was quantified by Thermo Scientific Pierce BCA Protein Assay Kit (Thermo Fisher Inc). All experiments were approved by the Office of Research Protection's Institutional Animal Care and Use Committee at Pennsylvania State University and by the National Institutes of Health. All experiments were performed in

accordance with the regulations of the Office of Research Protection's Institutional Animal Care and Use Committee at Cornell University and the National Institutes of Health.

3.4.2 Quantitative PCR. RNA was extracted either using TRIZOL reagent (Invitrogen) or using the RNAeasy kit (Invitrogen) and cDNA was generated with a kit from You Prime First-Strand beads kit (GE Biosciences). q-PCR was then performed with all values normalized to respective GAPDH values and the data expressed as 2^{-DDCt} (where Ct is threshold cycle), the values are represented as fold change over respective controls.

3.4.3 Flow cytometry and analysis of cytokines and transcription factors. Where indicated cells were stimulated with PMA/Ionomycin (Sigma) in the presence of Brefeldin A (Sigma) for 5 hours. Stimulated or unstimulated cells were fixed with Foxp3 fixation/ permeabilization kit (eBioscience) was used to fix/ permeabilize cells and then stained for the indicated surface proteins, and intracellular proteins. LSRII flow cytometer (BD Biosciences) was used to acquire data and the data was analyzed with FlowJo (TreeStar).

3.4.4 Data analysis. Statistical analysis was done with Student's *t* test, linear regression analysis, two-way ANOVA using GraphPad Prism version 5.00 for Windows (GraphPad, San Diego, CA). Differences with probability $p \leq 0.05$ were considered statistically significant.

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CHAPTER 4

$CD8^+$ T cells modulate the progression of Hypersensitivity Pneumonitis by regulating IL-17A expression in the lungs

4.1 Introduction

Hypersensitivity pneumonitis (also called extrinsic allergic alveolitis, EAA) is an inflammation of the alveoli within the lung caused by hypersensitivity to aerosolized antigen [1, 2]. HP is an environmental disease caused by repeated exposure to aerosolized antigens. In humans an example of HP, also called 'Farmer's lung,' is caused by thermophilic bacteria called *Saccharopolyspora rectivergula* (*S.rectivirgula*) due to repeated exposure to moldy hay [1-5]. HP occurs in three clinical forms acute, sub-acute and chronic with chronic patients suffering from pulmonary fibrosis and irreversible pulmonary dysfunction. In mice exposed to SR, the disease is characterized by lung fibrosis and mononuclear infiltrates mostly composed of macrophages and T cells [2, 6-8]. Recently it has been shown that peribronchovascular mononuclear infiltrates and resultant pulmonary fibrosis due to SR is caused by Th17 cells and IL-17A production [2]. $CD8^+$ T cells on the other hand have been shown to play a role in promoting inflammation during the acute stages of the disease [4, 9]. During acute inflammation, $CD8^+$ T cells interact with activated alveolar macrophages and secrete inflammatory mediators like IL-12, IL-2, IL-8 and IL-16. In humans, $CD8^+$ T cells have been shown to promote fibrosis and worsening of pulmonary function parameters with increased number of $CD8^+$ T cells correlating with exacerbated disease. Here, cytokines and chemokines produced by $CD8^+$ T cells directly and indirectly (through recruitment of other immune cells) increase collagen deposition and fibrosis. Also, like naïve $CD4^+$ T cells, naïve $CD8^+$ T cells have been shown to secrete IL-17A when cultured in the presence of TGF-β and IL-6 [10]. However, $CD8^+$ T cells may have minimal role in the development of pulmonary fibrosis in the chronic stages of *S. rectivirgula* mediated

hypersensitivity pneumonitis in mice as adoptive transfer of $CD8^+$ T cells into $TCR\beta^{-/-}\delta^{-/-}$ hosts followed by *S. rectivirgula* treatment resulted in minimal accumulation of $CD8^+$ T cells in the lungs and reduced collagen deposition compared to hosts that received $CD4^+$ T cells[2]. While, this suggests that $CD8^+$ T cells may not directly regulate pathology during chronic stages of hypersensitivity pneumonitis, whether $CD8^+$ T cells interact with other immune cell subsets to influence disease pathophysiology has not been investigated. Here we show that, depletion of $CD8^+$ T cell post induction of hypersensitivity pneumonitis did not affect the recruitment and/or maintenance of $CD4^+$ T cells in the lungs and bronchoalveolar regions but enhanced Th17 responses in both resulting in increased mononuclear infiltrates in the lungs. In line with this, $CD8^{-/-}$ mice show increased susceptibility to *S. rectivirgula* challenge with mice succumbing to disease as early as 2 weeks post *S. rectivirgula* challenge. These results suggest that $CD8^+$ T cells negatively regulate Th17 responses control against excessive inflammation against *S. rectivirgula.*

4.2 Results

4.2.1 Depletion of $CD8^+$ T cells during chronic hypersensitivity pneumonitis does not affect the accumulation of $CD4^+$ T cells in the lungs

$CD4^+$ T cells are the major contributor of lung pathology in hypersensitivity pneumonitis, and the number of $CD4^+$ T cells positively correlates with increased disease severity. Since the role of $CD8^+$ T cells in acute inflammation has been well established, we wanted to determine if $CD8^+$ T cells influence disease outcome during the chronic stages. To do this, we challenged mice with *S. rectivirgula* for 3 weeks (3 consecutive days per week) and depleted $CD8^+$ T cells after the induction of hypersensitivity pneumonitis (**Fig. 1**). Administration of α-CD8 antibody resulted in a significant reduction in the percentage and number of $CD8^+$ T cells in the lungs (**Fig. 2A**) as well as in the spleen and lymph nodes (**Fig. 2B**). Depletion of $CD8^+$ T cells had no effect on the accumulation of $CD4^+$ T cells in the lungs of *S. rectivirgula* challenged CD8 depleted versus non depleted mice (**Fig. 2C**). Similarly depletion of $CD8^+$ T cells had minimal effect on the accumulation of $CD4^+$ T cells in the bronchoalveolar lavage (**Fig. 2D**). This suggests that, $CD8^+$ T cells have no effect on the maintenance and/or recruitment of $CD4^+$ T cells during hypersensitivity pneumonitis.

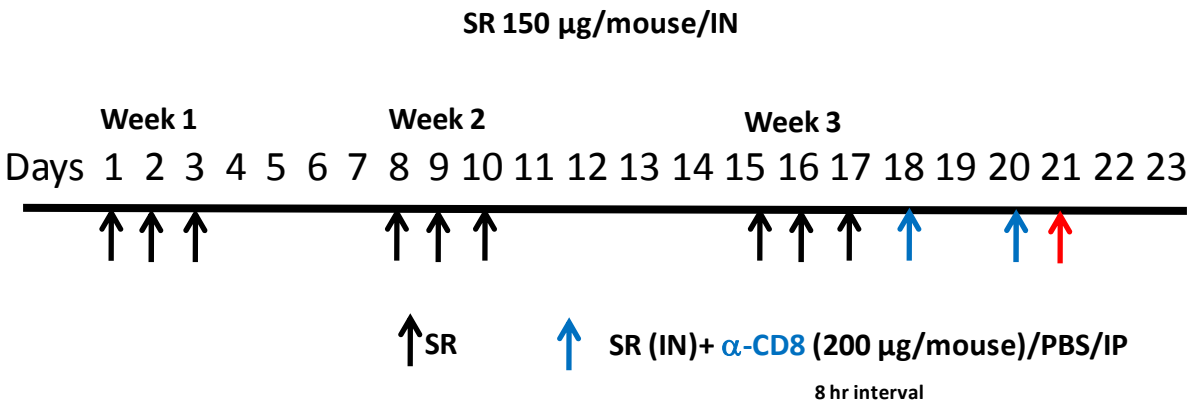
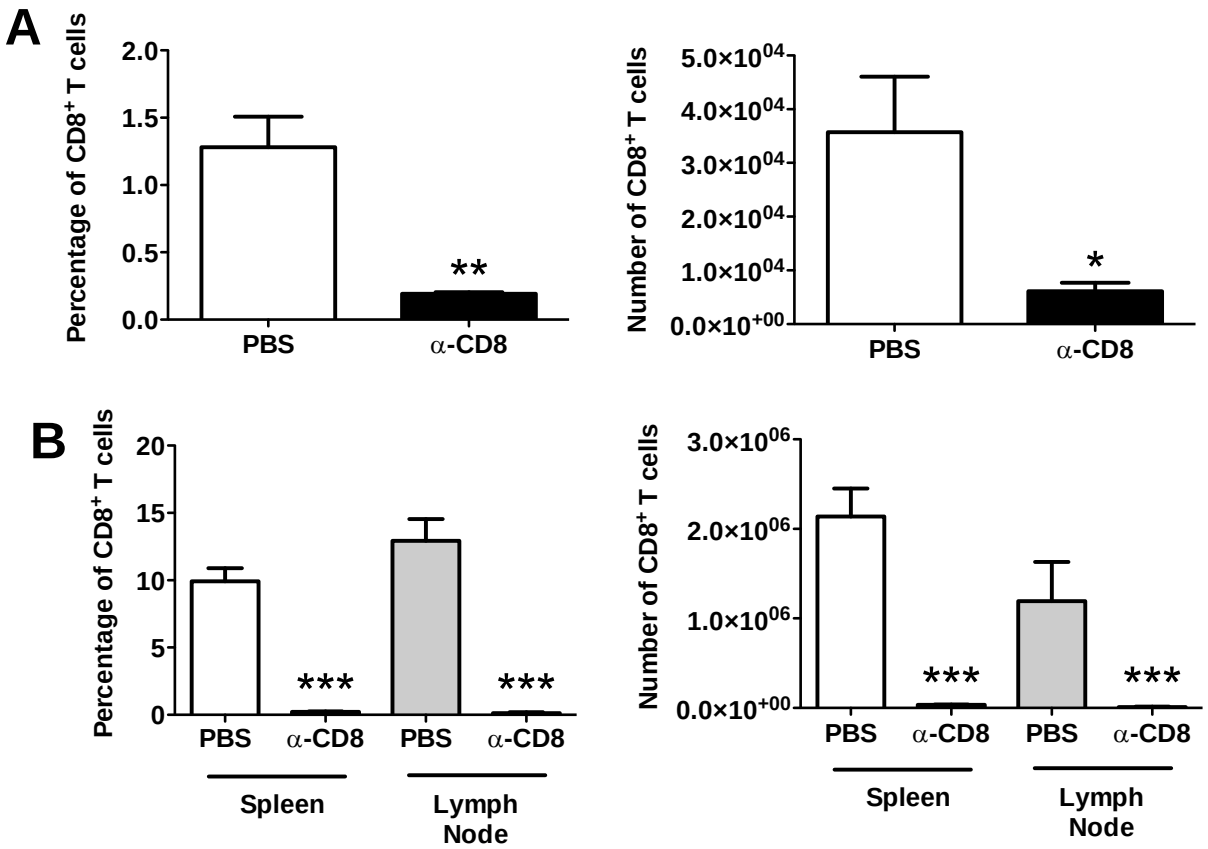


Fig.1 Schematic of *S. rectivirgula* challenge. Model diagram depicting the experimental layout for *S. rectivirgula* and CD8 depletion.



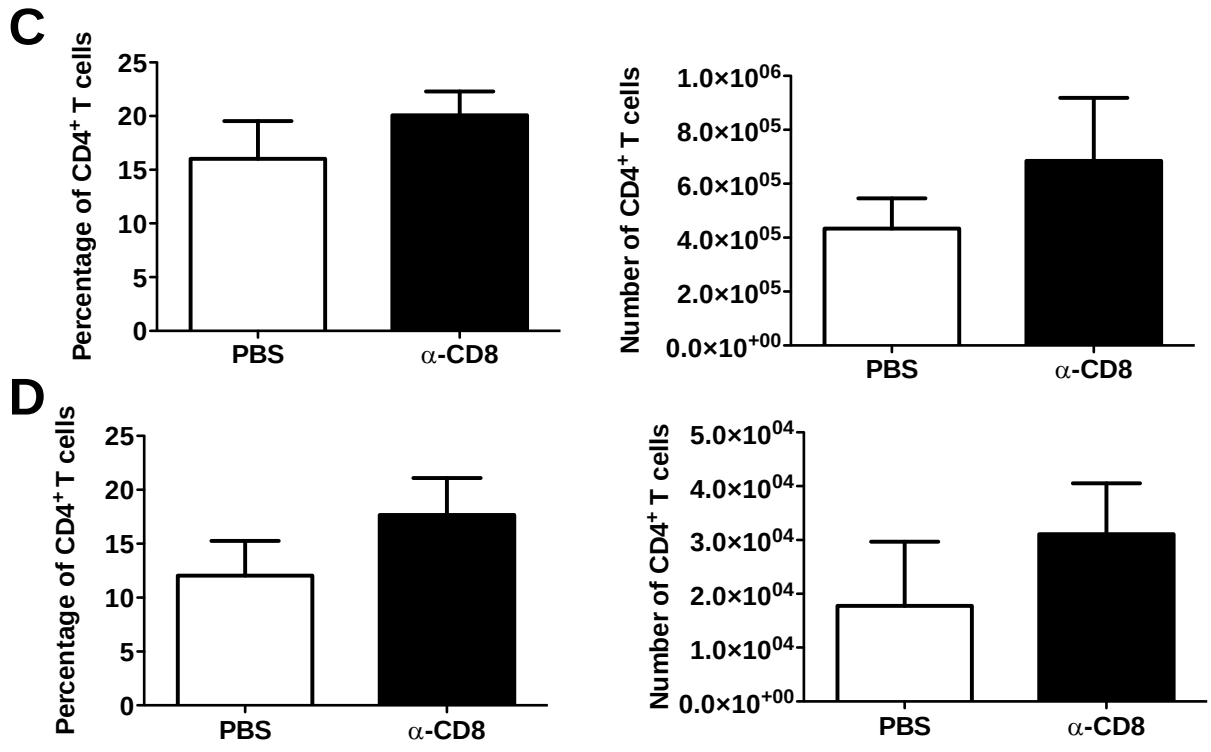


Fig.2 Depletion of CD8$^+$ T cells during chronic hypersensitivity pneumonitis does not affect the accumulation of CD4$^+$ T cells in the lungs. C57Bl/6 mice were intranasally exposed with *S. rectivirgula* (150µg/mouse) and injected either with PBS or an antibody against CD8 (200 µg/mouse i.p.). Single cell suspensions obtained from lungs (A) and spleen/ lymph node (B) were stained for CD8$^+$ T cells and analyzed by Flow cytometry. Single cell suspensions obtained from lungs (C) and BALF (D) of mice treated as above were analyzed for the percentage of infiltrating CD4$^+$ T cells by flow cytometry. Data represents Mean±SEM of n≥5 mice, *p<0.05 by unpaired students *t* test.

4.2.2 Depletion of CD8$^+$ T cells, post hypersensitivity pneumonitis induction, results in increased expression of IL-17A and IL-17F transcripts in the lungs.

In line with published results, challenge with *S. rectivirgula* resulted in a strong induction of IL-17A transcripts within the lungs (**Fig. 3A**). Interestingly, depletion of CD8$^+$ T cells

following the induction of chronic inflammation further increased the level of IL-17A within the lungs (**Fig. 3B**). Depletion of $CD8^+$ T cells also resulted in an increase in the levels of IL-17F, a cytokine that is functionally similar but less potent as compared to IL-17A (**Fig. 3C**). Depletion of $CD8^+$ T cells during the chronic stage, had minimal effect on the level of IFN-γ transcripts (**Fig. 3D**), another cytokine that has been shown to play a role in disease pathology during hypersensitivity pneumonitis. These results suggest that depletion of $CD8^+$ T cells can promote IL-17A mediated inflammation in the airways upon *S. rectivirgula* challenge.

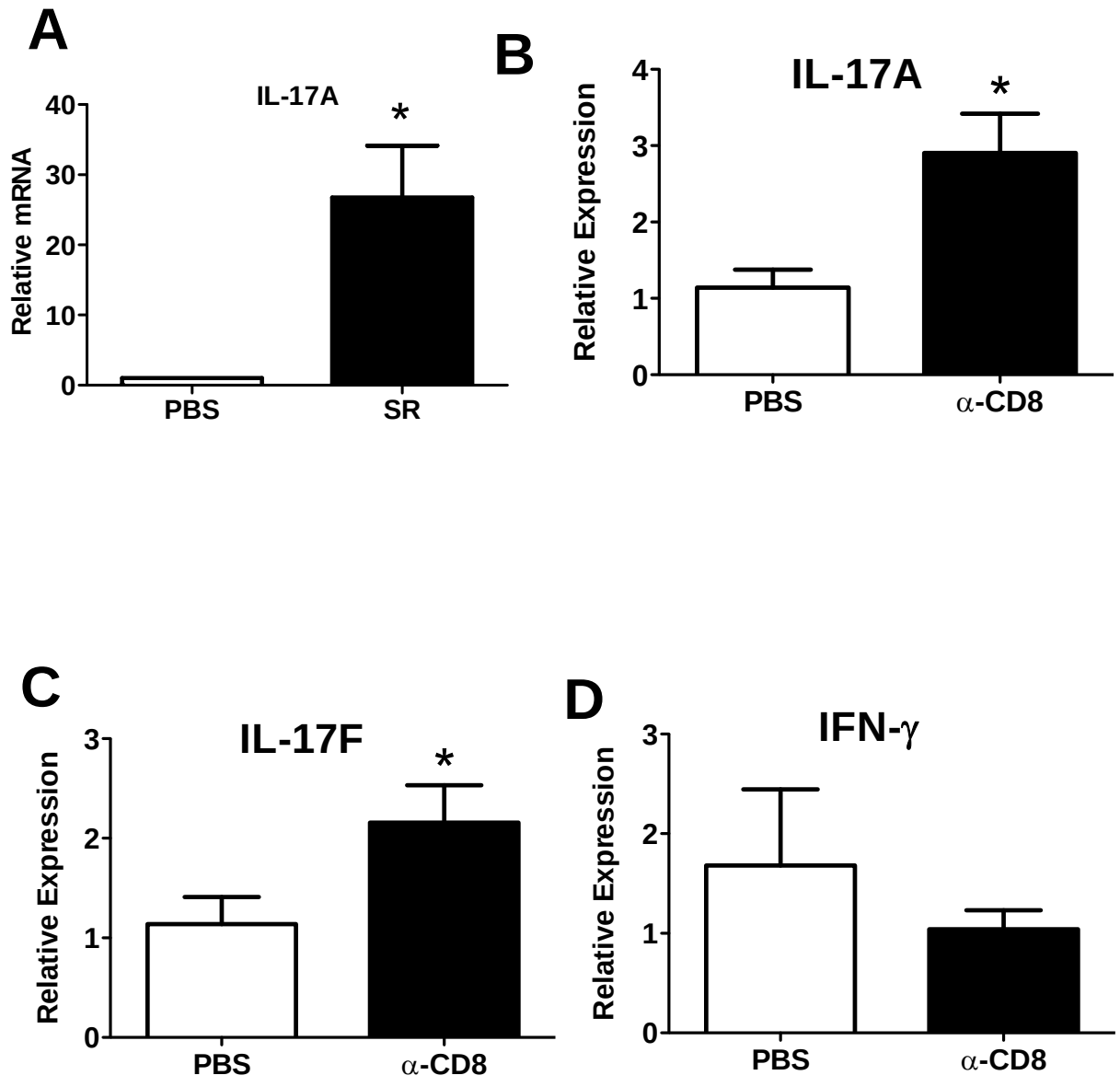


Fig.3 Depletion of CD8$^+$ T cells, post hypersensitivity pneumonitis induction, results in increased expression of IL-17A and IL-17F transcripts in the lungs. A) C57Bl/6 mice were intranasally exposed with *S. rectivirgula* (150µg/mouse) or PBS and RNA isolated from total lung tissue analyzed for the expression of IL-17A by qPCR. B-D) *S. rectivirgula* challenged mice were injected either with PBS or an antibody against CD8 (200 µg/mouse i.p.). QPCR analysis of RNA isolated from total lung tissue was analyzed for IL-17A,

IL-17F and IFNγ genes. Data represents Mean±SEM of n≥5 mice, *p<0.05 by unpaired students *t* test.

4.2.3 Depletion of $CD8^+$ T cells, post hypersensitivity pneumonitis induction, results in increased number of pathogenic Th17 cells in the lungs.

Th17 polarized $CD4^+$ T cells have been shown to play an important role in the development of inflammation and pulmonary fibrosis in *S. rectivirgula* challenged mice. Since we observed an increase in the levels of IL-17A and IL-17F transcripts in the lungs of *S. rectivirgula* challenged mice upon depletion of $CD8^+$ T cells, we wanted to determine if the loss of $CD8^+$ T cells had any effect on number of IL-17A producing $CD4^+$ T cells. We find that in the absence of $CD8^+$ T cells there was increased accumulation of IL-17A producing $CD4^+$ T cells both in the lungs (**Fig. 4A**) and in the bronchoalveolar lavage (**Fig. 4B**). Depletion of $CD8^+$ T cells neither affected the number of Treg cells nor the number of IL-10 producing $CD4^+$ T cells (**Fig. 4C**), suggesting that $CD8^+$ T cells regulated Th17 responses independent of regulatory $CD4^+$ T cells. In line with the fact that, we did not see a difference in IFN-γ transcript levels, depletion of $CD8^+$ T cells did not affect the number of IFN-γ^+ $CD4^+$ T cells in the lungs (**Fig. 4D**) and in the bronchoalveolar lavage (**Fig. 4E**). These results suggest that, $CD8^+$ T cells negatively regulate Th17 responses during the chronic stages of hypersensitivity pneumonitis.

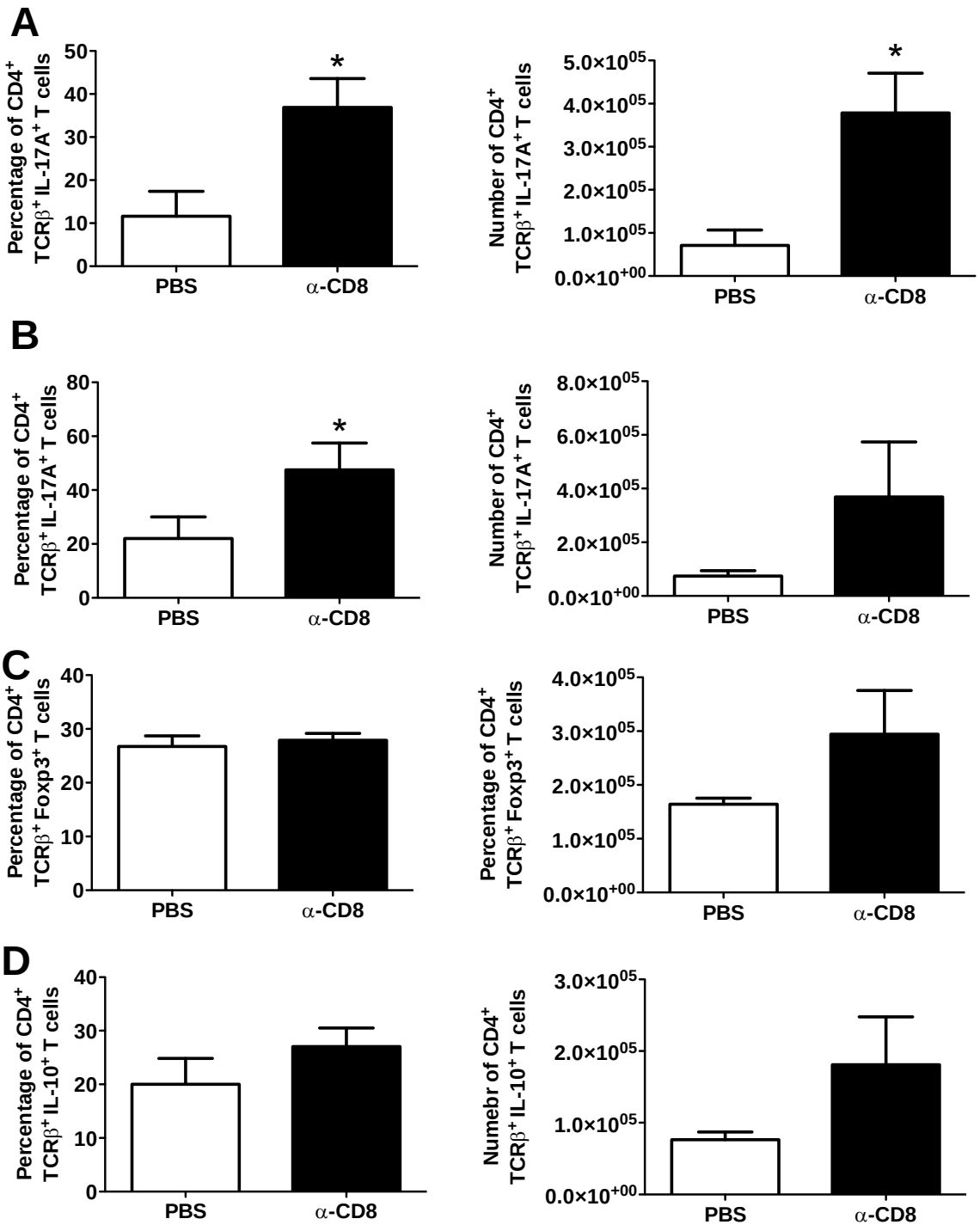


Fig.4 Depletion of CD8$^+$ T cells, post hypersensitivity pneumonitis induction, results in increased number of pathogenic Th17 cells in the lungs. C57Bl/6 mice

were treated with *S. rectivirgula* (150µg/mouse) and injected either with PBS or an antibody against CD8 (200 µg/mouse i.p.). Single cell suspensions obtained from lungs (A) and BALF (B) were stimulated with PMA and Ionomycin in the presence of Brefeldin A and analyzed for the percentage of IL-17A$^+$ producing CD4 T cells. C) Single cell suspensions obtained from lungs of mice treated as above were analyzed for the percentage of CD4$^+$ Foxp3$^+$ T cells and CD4$^+$ IL10$^+$ cells. D&E) Single cell suspensions obtained from lungs (D) and BALF (E) of mice treated as above were stimulated with PMA and Ionomycin in the presence of Brefeldin A and analyzed for the percentage of IFN-g$^+$ producing CD4 T cells. Data represents Mean±SEM of n≥5 mice, *p<0.05 by unpaired students *t* test.

4.2.4 Absence of CD8$^+$ T cells exacerbates inflammation in the lungs during hypersensitivity pneumonitis

Consistent with our findings that there were more Th17 cells in the lungs in the absence of CD8$^+$ T cells, H&E staining of lung sections from CD8$^+$ T cell depleted mice revealed increased number of peribronchovascular mononuclear infiltrates. Also, there was higher bronchiolar lumen occlusion in the CD8$^+$ T cell depleted mice compared to non-depleted controls (**Fig. 5A**).To further confirm the role of CD8$^+$ T cells in the regulation of hypersensitivity pneumonitis, we challenged CD8$^{-/-}$ mice with *S. rectivirgula* and found that these mice exhibit an exacerbated pathophysiology as shown by the fact that there was increased mononuclear infiltrates in the lungs (**Fig. 5B**). *S. rectivirgula* CD8$^{-/-}$ mice were also increasingly susceptible with mice succumbing to disease as early as 2 weeks post challenge (data not shown), which suggest that CD8$^+$ T cells are important for survival and an optimal immune response to *S. rectivirgula*. To date, Th17 polarized CD4$^+$ T cells have been shown to be the major contributor of hypersensitivity

pneumonitis, here we show that $CD8^+$ T cells play an important role in regulating the severity of the disease.

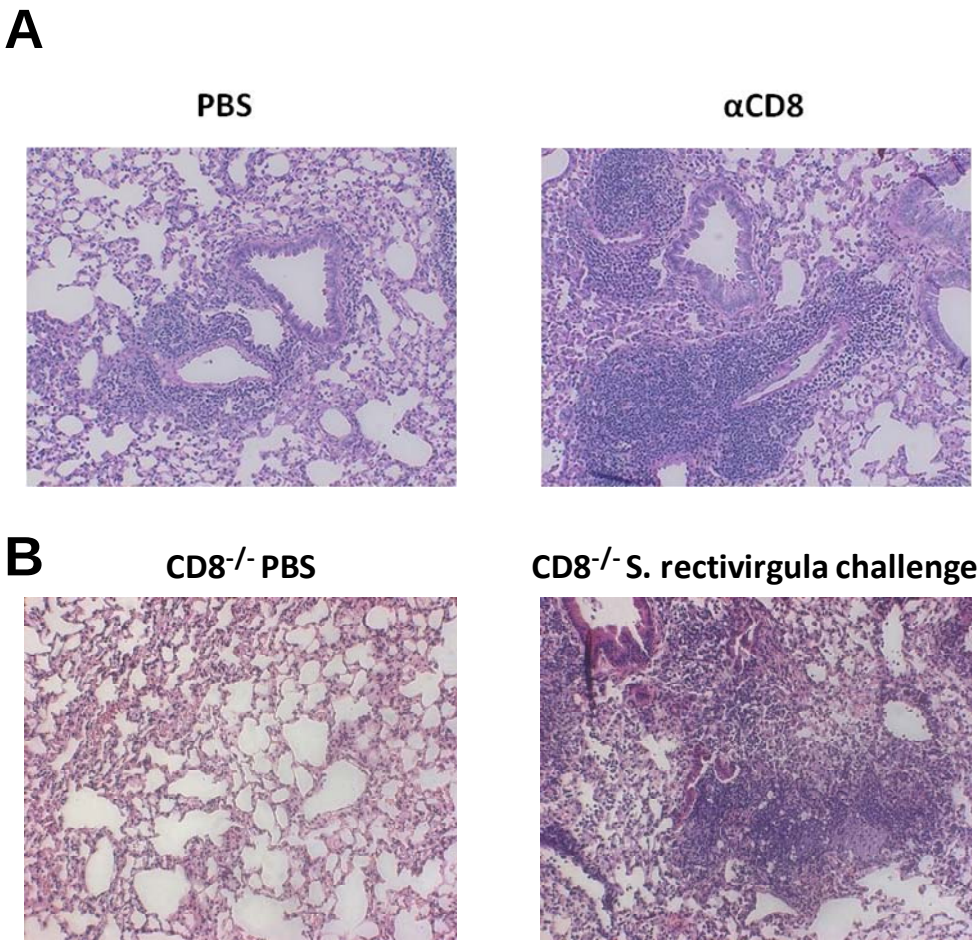


Fig. 5. Absence of $CD8^+$ T cells exacerbates inflammation in the lungs during hypersensitivity pneumonitis. A) C57Bl/6 mice were treated with *S. rectivirgula* (150µg/mouse) and injected either with PBS or an antibody against CD8 (200 µg/mouse i.p Lung sections were stained for H & E and analyzed at a magnification of 2.5x. B) C57Bl/6 or $CD8^{-/-}$ mice were treated with *S. rectivirgula* (150µg/mouse) and lung sections were stained for H & E and analyzed at a magnification of 20x.

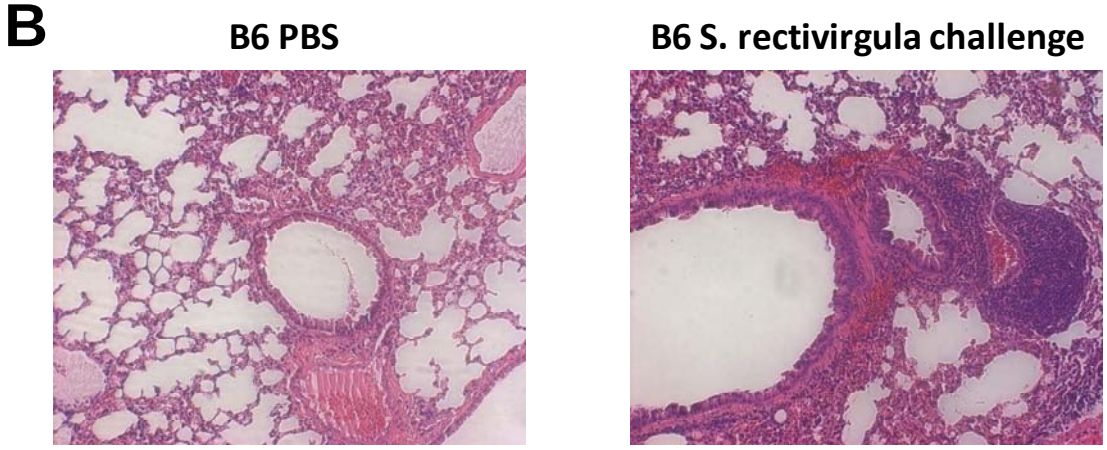


Fig. 5. Absence of $CD8^+$ T cells exacerbates inflammation in the lungs during hypersensitivity pneumonitis. A) C57Bl/6 mice were treated with *S. rectivirgula* (150µg/mouse) and injected either with PBS or an antibody against CD8 (200 µg/mouse i.p Lung sections were stained for H & E and analyzed at a magnification of 2.5x. B) C57Bl/6 or $CD8^{-/-}$ mice were treated with *S. rectivirgula* (150µg/mouse) and lung sections were stained for H & E and analyzed at a magnification of 20x.

4.3 Discussion

Hypersensitivity pneumonitis is a chronic inflammatory disease that is mediated by repeated exposure to certain environmental antigens and can result in collagen deposition leading to fibrosis in the lungs [11]. Antigens from the thermophile *S. rectivirgula* are one such class of environmental antigens that can cause hypersensitivity pneumonitis upon repeated exposure both in humans and mice [1, 3, 5, 12]. Inflammation in the chronic stages of *S. rectivirgula* mediated hypersensitivity pneumonitis is mainly driven by IL-17A producing $CD4^+$ T cells, as there is significantly lower inflammation and lung fibrosis both in the absence of $\alpha\beta$ T cells or in the absence of IL-17A receptor signaling [2]. Although, $CD8^+$ T cells play a role during the acute stages of the disease [4, 9], adoptive transfer experiments have suggested that $CD8^+$ T cells play minimal role in the pathogenesis when transferred by themselves during the chronic stages of hypersensitivity pnenumonitis [2]. However, whether $CD8^+$ T cells promote or control responses of other immune cell subsets to *S. rectivirgula* has not been investigated. In the absence of $CD8^+$ T cells during the chronic stages of hypersensitivity pneumonitis there is a marked increase in the infiltration and/ or expansion of Th17 cells, which resulted in an increase in mononuclear infiltrates within the lungs. Also, upon chronic exposure to *S. rectivirgula* $CD8^{-/-}$ mice had increased mononuclear infiltrates and pathology in the lungs. These results underscore a previously unappreciated role for $CD8^+$ T cells in the regulation of Th17 responses in the lungs.

Recently it has been shown that under chronic inflammatory settings such as EAE, IL-17A producing $CD8^+$ T cells can promote Th17 responses. Importantly this help

provided by $CD8^+$ T cell in enhancing $CD4^+$ T cell responses were contingent upon secretion of IL-17A by $CD8^+$ T cells and is mediated by a cell-cell interaction between $CD8^+$ and $CD4^+$ T cells [10]. This is supported by the fact that, $CD8^{-/-}$ mice have lower disease pathology during EAE and depletion of $CD8^+$ T cells with neutralizing CD8 antibody resulted in less severe disease [10, 13]. This promotion of Th17 responses by $CD8^+$ T cell was further enhanced under lymphopenic environment [10]. Although these results provide a strong evidence for enhancement of Th17 responses by IL-$17A^+$ $CD8^+$ T cells, it is not clear if this holds true for other subsets of $CD8^+$ T cells and/ or during different chronic inflammatory settings. Our results, suggest that control of $CD4^+$ T cell responses by $CD8^+$ T cells is much more complex and is dependent on the inflammatory setting. As in the case of the chronic inflammatory disease hypersentivity pneumonitis, where there is very minimal to no induction of IL-$17A^+$ $CD8^+$ T cells (data not shown), absence of $CD8^+$ T cells resulted in a more exaggerated Th17 response resulting in more severe pathology. This to our knowledge is the first report suggesting that under certain chronic inflammatory settings $CD8^+$ T cells can inhibit Th17 responses to curb excessive inflammation. It would be interesting to see if this control of $CD4^+$ T cell response is dependent on cell-cell interaction and what are the other factors that govern $CD8^+$ T cell mediated control versus promotion of Th17 response.

4.4 Materials and Methods

4.4.1 Mice and treatments. C57bl/6 mice were purchased from Jackson laboratories. All mice were used between 6- and 12-wk of age and maintained in a specific pathogen free environment. For hypersentivity pneumonitis experiments B6 mice were given 150µg (50µl) of *S. rectivirgula* (American Type Tissue Collection catalogue no. 29034) or sterile 1X PBS. Mice were lightly anesthetized with isoflurane and either *S.rectivirgula* or sterile PBS was administered intranasally for 3 consecutive days for 3 weeks. *S. rectivirgula* was prepared by growing the microorganism in tryptic soy broth at 55°C with constant agitation. The *S. rectivirgula* culture was centrifuged, resuspended in sterile PBS, and the concentration of the SR protein was quantified by Thermo Scientific Pierce BCA Protein Assay Kit (Thermo Fisher Inc). All experiments were approved by the Office of Research Protection's Institutional Animal Care and Use Committee at Pennsylvania State University and by the National Institutes of Health. All experiments were performed in accordance with the regulations of the Office of Research Protection's Institutional Animal Care and Use Committee at Cornell University and the National Institutes of Health.

4.4.2 Quantitative PCR. RNA was extracted either using TRIZOL reagent (Invitrogen) or using the RNAeasy kit (Invitrogen) and cDNA was generated with a kit from You Prime First-Strand beads kit (GE Biosciences). q-PCR was then performed with all values normalized to respective GAPDH values and the data expressed as 2^{-DDCt} (where Ct is threshold cycle), the values are represented as fold change over respective controls.

4.4.3 Flow cytometry and analysis of cytokines and transcription factors. Where indicated cells were stimulated with PMA/Ionomycin (Sigma) in the presence of Brefeldin A (Sigma) for 5 hours. Stimulated or unstimulated cells were fixed with Foxp3 fixation/ permeabilization kit (eBioscience) was used to fix/ permeabilize cells and then stained for the indicated surface proteins, and intracellular proteins. LSRII flow cytometer (BD Biosciences) was used to acquire data and the data was analyzed with FlowJo (TreeStar).

4.4.4 Data analysis. Statistical analysis was done with Student's *t* test using GraphPad Prism version 5.00 for Windows (GraphPad, San Diego, CA). Differences with probability $p \leq 0.05$ were considered statistically significant

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Chapter 5

Summary

5.1 Summary

Subsets of $CD4^+$ T cells (Th1, Th2, Th17 and Treg) play a vital role in adaptive immune responses, regulating both inflammatory and anti-inflammatory processes, providing both immune protection as well as mediating immune pathology. Modulation of AHR signaling has been shown to regulate differentiation of all these four T helper cell subsets, but recently much emphasis has been placed on its role in regulation of Th17 and Treg responses as AHR is believed to be a potential link in the observed interplay between environmental stress and autoimmune diseases [1-6]. Th17 cells are a subset of $CD4^+$ T cells that are required for clearance of extracellular pathogens and fungi and have been implicated in the development of a number of chronic immune-mediated diseases [7-9]. Th17 cells secrete IL-17A and IL-17F among six other IL-17 family cytokines and can coordinate with innate immune cells like neutrophils, inducing their recruitment to the site of inflammation for an integrated and optimal immune response [8, 10, 11]. Treg cells on the other hand are another subset of $CD4^+$ T cells that play a balancing role in controlling aberrant immune responses that includes control against autoimmune and inflammatory pathologies [11, 12]. Thus, the regulation of Th17/Treg axis is critical for the control of inflammatory and autoimmune diseases [2, 3, 8, 13].

Aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor, which acts as an environmental sensor that can bind to ligands like polycyclic aromatic compounds (for example environmental pollutants and industrial byproducts) to induce toxic responses mediated by xenobiotic enzymes [6, 14, 15]. In addition, recent evidence points towards AHR signaling to play a critical role in regulation of both the innate and

adaptive immune responses [4, 15-20]. Along these lines, AHR signaling has been shown to regulate IL-17A and IL-22 secretion by innate lymphoid cells, agonists of AHR have been shown to induce tolerogenic DCs and also AHR has been identified as one of the factors that can regulate both Th17 and Treg cell differentiation [2, 3, 21-23]. Control of Th17/Treg cell axis by AHR is especially interesting, as it seems to do this in a ligand specific manner. TCDD or dioxin, the most potent AHR agonist, suppresses EAE by promoting differentiation of Treg cells while FICZ, also an agonist of AHR exacerbates EAE by promoting differentiation of Th17 cells, suggesting ligand dependent functions of AHR in controlling the Th17/Treg axis [2, 3, 24]. This phenomenon of ligand specific activation of AHR resulting in very different functional outcomes, can at least partly be explained by the fact that AHR can be activated by either the binding of ligand-AHR complex to its cognate response elements, dioxin response element (DRE) or through a DRE independent pathway. In this study, we have used three classes of AHR ligands, agonist of AHR (β-NF, TCDD and FICZ) that activates both the DRE- and non-DRE driven pathways, AHR antagonist (GNF) that inhibits both the DRE- and non-DRE and a selective AHR modulator (SGA-360), a partial antagonist, which would selectively activate the non-DRE driven responses without the induction of the DRE-mediated pathway. The study of SAHRMs for example, SGA-360 are interesting as they are useful tools to understand the differential role of DRE versus non-DRE driven responses in modulating Th17/Treg axis.

Here we show that, AHR can regulate IL-17A secretion by activated $CD4^+$ T cells under non-polarizing conditions. Under these conditions while agonists of AHR promoted IL-17A production, a partial antagonist inhibited it, suggesting that selective inhibition of

the DRE mediated pathway was sufficient to inhibit IL-17A both at the level of transcription and translation. Similarly, we find that regulation of Th17 differentiation is mediated by a DRE-mediated pathway, as both SGA-360 and GNF can efficiently inhibit differentiation of naïve $CD4^+$ T cells into Th17 cells. AHR has been shown to interact with Stat3 to regulate the expression of Aiolos, which can silence *Il2* locus to promote Th17 differentiation [25]. Also, IFNγ mediated activation of Stat1 can inhibit both TH17 and Treg differentiation. It has been shown that AHR is capable of interacting with Stat1 and Stat5 at least under Th17 polarizing conditions, preventing them from binding to the *Il17a* promoter and hence promoting IL-17A expression [26]. Based on this, it would be interesting to evaluate if inhibition of the DRE-mediated AHR pathway affects the interaction between these Stat proteins and AHR. Interestingly, suppression of Foxp3 expression during Th17 differentiation was mediated by a DRE-independent mechanism as only the complete antagonist GNF could reduce the expression of Foxp3 under these conditions. On the other hand, selective activation of the non-DRE mediated pathway by SGA-360 under Treg skewing conditions did not repress Foxp3 expression, but activation of the DRE- driven pathway was required to further enhance Foxp3 expression albeit in a ligand specific manner (TCDD and FICZ but not β-NF). GNF which blocks both the DRE and non-DRE driven pathways, significantly reduced the differentiation of naïve $CD4^+$ T cells into Tregs. It has been shown that upon activation by TCDD, AHR can directly bind to both the conserved AHR binding sites (CABS) and the non-evolutionarily conserved AHR binding sites (NCABS) in the *Foxp3* locus to regulate its expression [2]. The ability of the above mentioned ligands of AHR to activate or inhibit binding of AHR to these regions could dictate whether they promote or

inhibit Treg differentiation. Along these lines, it is imperative to study SAHRMs that can selectively activate the DRE response while inhibiting the non-DRE driven pathway, to further delineate the role of DRE versus non-DRE pathways in the regulation of Foxp3 expression. These results significantly adds to our current understanding of the role of AHR signaling in regulation of Th17 versus Treg cell differentiation as we provide a basis for the observed ligand specific function of AHR, differential activation of DRE versus non-DRE mediated pathways by ligands. Furthermore, it would be interesting to see if AHR signaling regulates signaling pathways that are known to differentially regulate effector versus regulatory $CD4^+$ T cell differentiation like the mTORC signaling pathway [27].

These results highlight the importance of AHR signaling in control of Th17/ Treg differentiation and underscore the complexity in AHR's regulation of these signaling events. The fact that AHR can also regulate the secretion of IL-22 by $CD4^+$ T cells, production of both IL-17A and IL-22 by innate lymphoid cells and promote differentiation of tolerogenic DC's *in vivo*, further adds to this complexity [28-33]. This is most apparent in the gut, where in spite of the fact that AHR has been shown to be a critical transcription factor for differentiation of Th17 cells, absence of AHR, as in the case of $AHR^{-/-}$ mice, promotes intestinal Th17 responses [2, 34]. This is in stark contrast to what is known about AHR signaling in pathogenesis during EAE, where the absence of AHR ameliorates disease progression [3]. Also, inhibition of the enzyme kynurenine 3-monooxygenase (KMO), which catabolizes endogenous ligands of AHR has been shown to promote Th17 responses to exacerbate autoimmune gastritis [35]. In addition to this, Th17 cell plasticity, which is defined by its ability to produce alternate Th lineage

cytokines, varies between acute and chronic inflammatory conditions [36]. Thus it is important to better understand how activation and inhibition of AHR signaling with AHR ligands would influence Th cell responses *in vivo* for further validation of AHR as a therapeutic target.

Using the acute *C. rodentium* mediated infection model (where Th17 is protective) and the chronic *S. rectivirgula* mediated hypersensitivity pneumonitis model (where Th17 is pathogenic) we show that activation of AHR by the agonist β-NF can promote both Th17 differentiation (*C. rodentium* model) and enhance IL-17A by fully differentiated Th17 cells (*S. rectivirgula* model). Inhibition of AHR on the other hand can inhibit Th17 differentiation but has minimal effect on the ability of fully differentiated Th17 cells to make IL-17A. It is interesting to note that in the chronic *S. rectivirgula* model, even though both the partial and complete antagonist has no effect on the IL-17A production, there was significant promotion of anti-inflammatory responses by increase in IL-4 (both in GNF and SGA-360) and an increase in the expression of Foxp3 (GNF) (**Fig1 & 2**).

This work has implications for understanding AHR as a therapeutic target for both acute and chronic inflammatory diseases in the context of regulation of pro- versus anti-inflammatory responses including effector versus regulatory T helper cell differentiation.

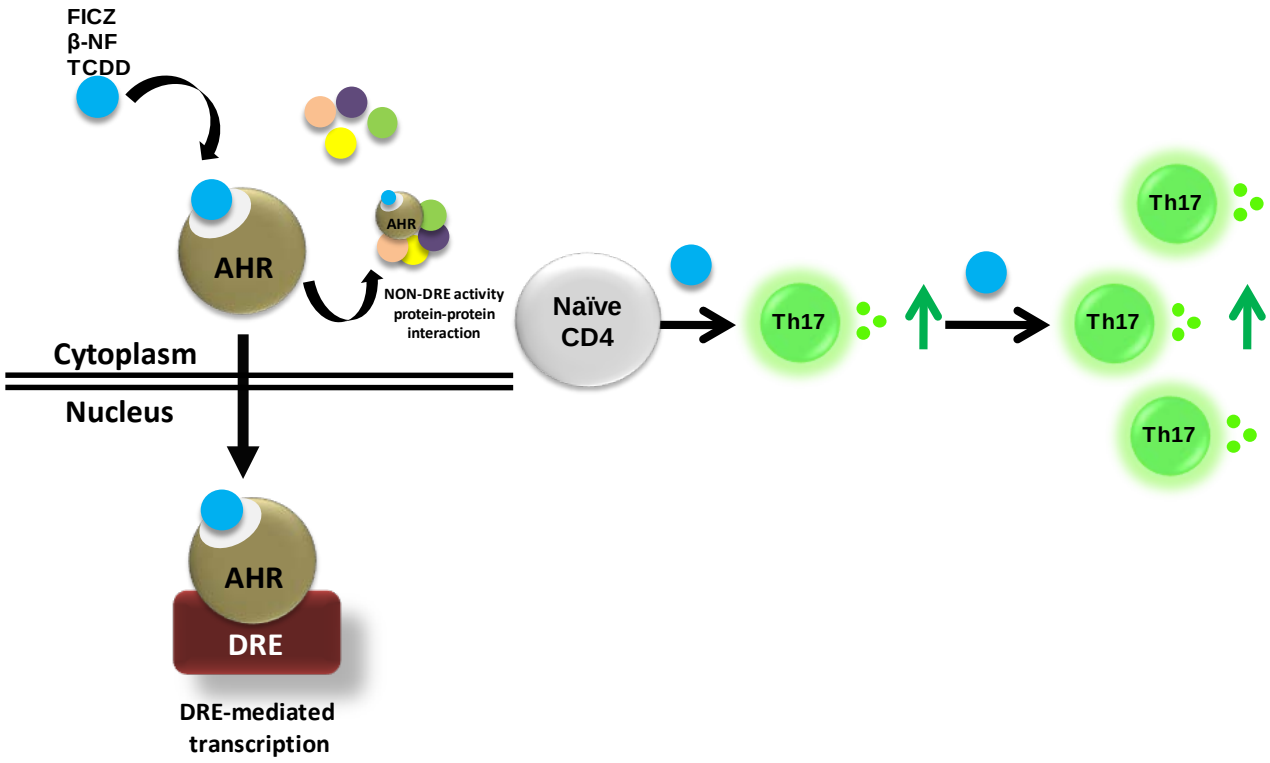

Fig.1. Effect of DRE- mediated activation on Th17 differentiation

Agonists (FICZ, β-NF, TCDD) of AHR can activate both the DRE and NON-DRE driven responses to promote Th17 differentiation from naïve $CD4^+$ T cells and on already differentiated Th17 cells it can further enhance Th17 differentiation.

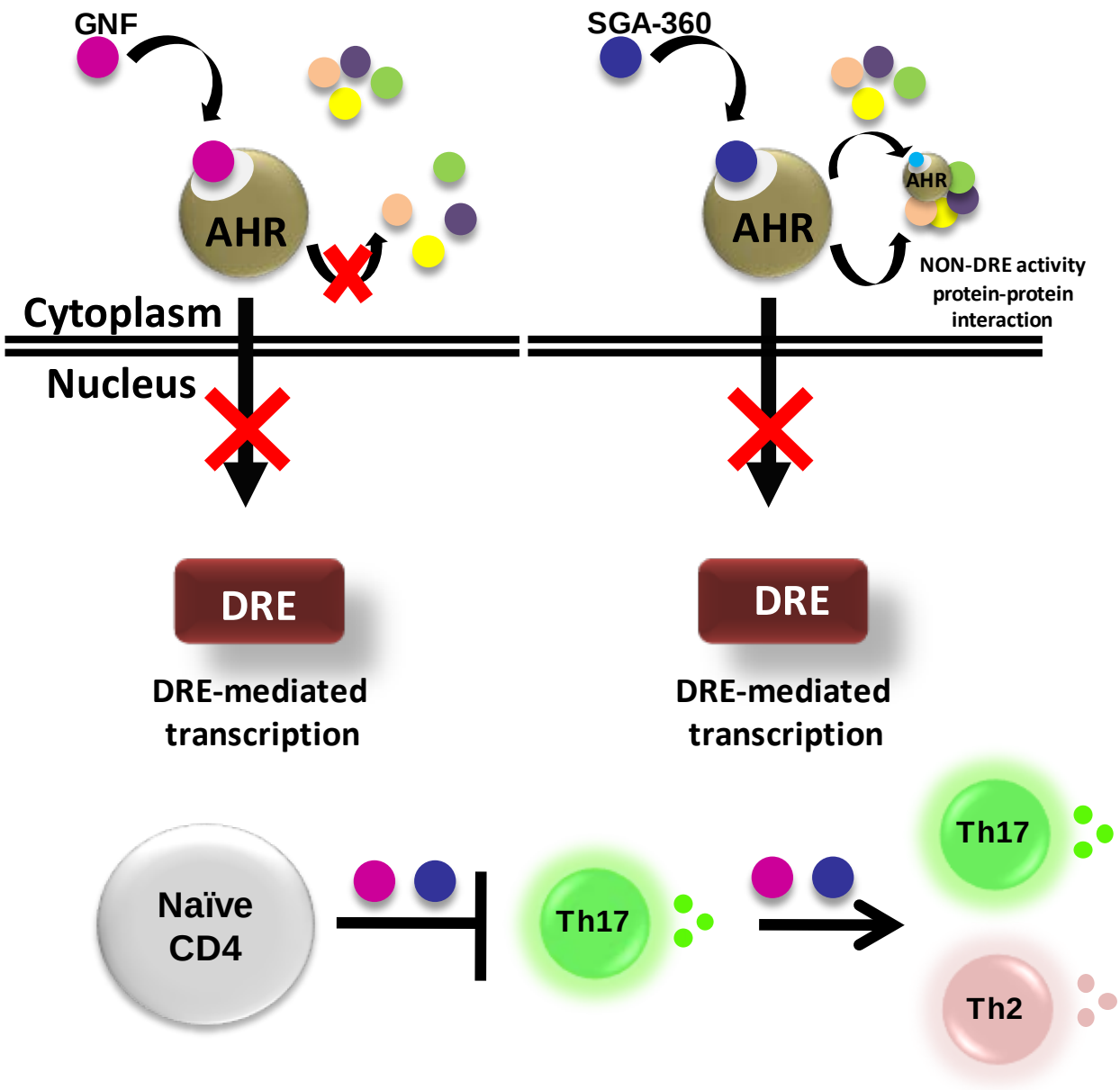

Fig. 2. Effect of DRE-mediated inhibition on Th17 differentiation

Both antagonists (GNF) and partial antagonist (SGA-360) can inhibit DRE driven responses and can suppress Th17 differentiation from naïve $CD4^+$ T cells and on already differentiated Th17 cells have minimal effect but can produce alternate lineage cytokines.

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